UNIVERSIDADE FEDERAL DO CEARÁ
CENTRO DE CIÊNCIAS
DEPARTAMENTO DE BIOQUÍMICA E BIOLOGIA MOLECULAR
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA

BRANDON FERRAZ E SOUSA

EFEITO DE PROTEÍNAS TERAPEUTICAS DO LÁTEX DE *Calotropis procera* EM ANIMAIS HIPERGLICÊMICOS INFECTADOS COM *Salmonella*: MODULAÇÃO DA RESPOSTA INFLAMATÓRIA E PROPRIEDADES REOLÓGICAS DO SANGUE

FORTALEZA
2024

BRANDON FERRAZ E SOUSA

EFEITO DE PROTEÍNAS TERAPEUTICAS DO LÁTEX DE *Calotropis procera* EM ANIMAIS HIPERGLICÊMICOS INFECTADOS COM *Salmonella*: MODULAÇÃO DA RESPOSTA INFLAMATÓRIA E PROPRIEDADES REOLÓGICAS DO SANGUE

Tese apresentada a Coordenação do Programa de Pós-Graduação em Bioquímica do Centro de Ciências da Universidade Federal do Ceará, como requisito parcial para obtenção do título de Doutor em Bioquímica.
Área de Concentração: Bioquímica
Orientador: Prof. Dr. Márcio Viana Ramos

FORTALEZA
2024

Dados Internacionais de Catalogação na Publicação
Universidade Federal do Ceará
Sistema de Bibliotecas
Gerada automaticamente pelo módulo Catalog, mediante os dados fornecidos pelo(a) autor(a)

S696e Sousa, Brandon Ferraz.
Efeito de proteínas terapêuticas do látex de Calotropis procera em animais hiperglicêmicos infectados com Salmonella: Modulação da resposta inflamatória e propriedades reológicas do sangue / Brandon Ferraz Sousa. – 2024.
60 f. : il. color.

Tese (doutorado) – Universidade Federal do Ceará, Centro de Ciências, Programa de Pós-Graduação em Bioquímica , Fortaleza, 2024.
Orientação: Prof. Dr. Márcio Viana Ramos.

1. citocinas. 2. imunomodulação. 3. Salmonella. 4. viscosidade. I. Título.

CDD 572

BRANDON FERRAZ E SOUSA

EFEITO DE PROTEÍNAS TERAPEUTICAS DO LÁTEX DE *Calotropis procera* EM ANIMAIS HIPERGLICÊMICOS INFECTADOS COM *Salmonella*: MODULAÇÃO DA RESPOSTA INFLAMATÓRIA E PROPRIEDADES REOLÓGICAS DO SANGUE

Tese apresentada a Coordenação do Programa de Pós-Graduação em Bioquímica do Centro de Ciências da Universidade Federal do Ceará, como requisito parcial para obtenção do título de Doutor em Bioquímica.
Área de Concentração: Bioquímica

Aprovado em: ___/___/_____

BANCA EXAMINADORA

_____

Dr. Márcio Viana Ramos (Orientador)
Universidade Federal do Ceará

_____

Dra. Nylane Maria Nunes de Alencar
Universidade Federal do Ceará

_____

Dr. Jeanlex Soares de Sousa
Universidade Federal do Ceará

_____

Dr. Felipe Domingos de Sousa
Universidade de Fortaleza

_____

Dra. Ayrles Fernanda Brandão Silva
Perícia Forense do Ceará

AGRADECIMENTOS

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

Expresso minha sincera gratidão a todos os veneráveis colegas e mentores que, com sua sabedoria e dedicação, acompanharam-me na jornada quaternária que culminou na gênese desta tese. Um tributo especial ao Prof. Dr. Márcio Viana Ramos, cuja erudição e orientação foram faróis a iluminar o caminho deste aprendizado. Minhas estimadas colegas de laboratório, Marina, Larissa, Sara e Dureshahwar, merecem meu reconhecimento por sua inestimável colaboração técnica e apoio emocional, pilares que sustentaram o desenvolvimento deste trabalho.

À minha família, meu porto seguro, ofereço meu amor e gratidão por seu suporte inabalável e fé incondicional em minha jornada, livre de qualquer juízo. Aos meus amigos, estendo meu agradecimento por emprestarem seus ouvidos atentos aos meus desabafos e por compartilharem os altos e baixos desta trajetória. Gerlane, Gerval, Deborah, Sheila, Adriana, Wallady, Pedro, Léa, Jenniffer e Andressa.

E não poderia deixar de enaltecer as tias das cantinas da UFC, cujas mãos prepararam os manjares que nutriram não apenas nossos corpos, mas também nossas almas, em meio a conversas e camaradagem.

RESUMO

A diabetes mellitus (DM), uma doença crônica que afeta milhões de pessoas globalmente, caracteriza-se pela prevalência de níveis elevados de glicose no sangue (hiperglicemia) e está associada a um maior risco de diversas complicações, incluindo infecções. A busca por novas abordagens terapêuticas para o diabetes e suas complicações é constante. As proteínas do látex da planta *Calotropis procera* (CpLP), com diversas atividades e modulações farmacológicas, relatadas, poderiam ser um potencial agente promissor nesta condição fisiológica adversa. A hiperglicemia foi induzida em camundongos com uso de streptozotocina (i.p). Na primeira etapa do estudo, a fim de avaliar o impacto de CpLP na sobrevivência e na resposta inflamatória, os animais hiperglicêmicos (glicemia maior que 220 mg/ dL) foram desafiados com uma infecção bacteriana letal, de *Salmonella* Typhimurium, que foi definida para o modelo de camundongo hiperglicêmico, como 10^4 CFU/ mL. A sobrevivência dos animais foi monitorada diariamente, durante 7 dias para determinação de diferenças significativas entre os grupos. Amostras de sangue e fluido peritoneal foram coletadas após 24 e 72 horas de infecção para análise detalhada dos níveis das citocinas TNF-α, IL-1β e IL-10. Fígado, rins e pâncreas foram coletados para avaliação histopatológica relacionadas à inflamação e hiperglicemia. Na segunda etapa do estudo, a reologia e hematologia, do sangue dos camundongos, foram analisadas. A viscosidade sanguínea, um indicador importante da fluidez do sangue, foi medida utilizando reometria rotacional e analisada por meio de modelo matemático de fluido de Bingham. Parâmetros hematológicos, como contagem de glóbulos vermelhos, brancos e plaquetas, hematócrito e hemoglobina, foram avaliados em um hemograma completo. O tratamento com CpLP nas doses de 30 e 60 mg/ kg resultou em um aumento na sobrevida dos camundongos hiperglicêmicos infectados, em comparação com o grupo controle. No entanto, CpLP não foi capaz de evitar a morte dos camundongos infectados. A análise das citocinas revelou que a CpLP modulou a resposta inflamatória, reduzindo os níveis das citocinas pró-inflamatórias TNF-α e IL-1β no fluido peritoneal e plasmático após a infecção. O tratamento também elevou os níveis da citocina anti-inflamatória IL-10, sugerindo um efeito anti-inflamatório, mesmo na condição hiperglicemiante. No plasma, a CpLP apresentou um efeito similar sobre o TNF-α, mas não afetou os níveis de IL-1β ou IL-10. Na reologia do

sangue, a hiperglicemia aumentou a viscosidade sanguínea, mas o tratamento com CpLP tornou o sangue menos viscoso e facilitou o seu fluxo, tanto em animais infectados e não, revertendo o comportamento fluídico para parâmetros similares ao de animais não hiperglicêmicos. Esses resultados sugerem que o tratamento com CpLP equilibrou as respostas pró-inflamatórias e anti-inflamatórias e reduziu o dano inflamatório causado pela infecção, mesmo não prevenindo a morte. Além disso, esses resultados demonstram o potencial terapêutico de CpLP em reverter alterações reológicas no sangue, causadas pela hiperglicemia.
Palavras-chave: citocinas; imunomodulação; *Salmonella*; viscosidade;

ABSTRACT

Diabetes mellitus (DM), a chronic disease that affects millions of people globally, is characterized by the prevalence of high blood glucose levels (hyperglycemia) and is associated with an increased risk of various complications, including infections. The search for new therapeutic approaches to diabetes and its complications is constant. Latex proteins from the plant *Calotropis procera* (CpLP), with various reported pharmacological activities and modulations, could be a promising potential agent in this adverse physiological condition. Hyperglycemia was induced in mice using streptozotocin (i.p). In the first stage of the study, in order to assess the impact of CpLP on survival and the inflammatory response, the hyperglycemic animals (blood glucose greater than 220 mg/ dL) were challenged with a lethal bacterial infection of *Salmonella* Typhimurium, which was defined for the hyperglycemic mouse model as 10^4 CFU/ mL. The survival of the animals was monitored daily for 7 days to determine any significant differences between the groups. Blood and peritoneal fluid samples were collected 24 and 72 hours after infection for detailed analysis of the levels of the cytokines TNF-α, IL-1β and IL-10. Liver, kidneys and pancreas were collected for histopathological evaluation ~~related to~~ inflammation and hyperglycemia. In the second stage of the study, the rheology and hematology of the mice's blood were analyzed. Blood viscosity, an important indicator of blood fluidity, was measured using rotational rheometry and analyzed using a mathematical Bingham fluid model. Hematological parameters, such as red blood cell, white blood cell and platelet counts, hematocrit and hemoglobin, were evaluated in a complete blood count. Treatment with CpLP at doses of 30 and 60 mg/ kg resulted in an increase in the survival of infected hyperglycemic mice compared to the control group. However, CpLP was unable to prevent the death of the infected mice. Cytokine analysis revealed that CpLP modulated the inflammatory response, reducing the levels of the pro-inflammatory cytokines TNF-α and IL-1β in the peritoneal fluid and plasma after infection. Treatment also raised levels of the anti-inflammatory cytokine IL-10, suggesting an anti-inflammatory effect, even in the hyperglycemic condition. In plasma, CpLP showed a similar effect on TNF-α, but did not affect IL-1β or IL-10 levels. In blood rheology, hyperglycemia increased blood viscosity, but treatment with CpLP made the blood less viscous and facilitated its flow, both in infected and non-infected animals, reverting fluid behavior to parameters similar to those of non-

hyperglycemic animals. These results suggest that treatment with CpLP balanced the pro-inflammatory and anti-inflammatory responses and reduced the inflammatory damage caused by the infection, even though it did not prevent death. Furthermore, these results demonstrate the therapeutic potential of CpLP in reversing rheological changes in the blood caused by hyperglycemia.

Keywords: cytokines; immunomodulation; *Salmonella*; viscosity;

SUMMARY

1 INTRODUCTION

1.1 Natural products and latex

Biodiversity is the main source of natural resources of biological importance. Medicinal plants can be used in fabrication of phytotherapics and phytopharmaceuticals. The identification of bioactive molecules in plants lead to the discovery of new chemicals and development of biotechnological inputs and drugs. Among these bioactive molecules, proteins are the main target, due to their relevant activity and relative easiness to obtain (Guerra; Nodari, 2004).

The use of natural products and biotechnological agents has evolved alongside the advances in screening technologies. There are many drugs broadly used in medicinal treatment, that were obtained and/or produced from plants, such as penicillin and morphine (Li *et al.*, 2019).

Latex is an endogenous fluid, produced in specific cells called laticifers. It is composed of various substances, such as terpenoids, proteins, sugars, fats and many other metabolites. This fluid is exuded when parts of the plant are damaged, releasing the latex containing not only the molecules said above, but rubber (polyisoprene). Many of the molecules comprising the latex have been studied for their activity, and many results support the theory of the role of latex in plant defense. The action-specificity of these enzymes makes them a very interesting target for pharmacological studies (Ramos *et al.*, 2019).

1.2 *Calotropis procera*

Calotropis procera (Aiton) W. T. Aiton, is an angiosperm from Apocynaceae family. It is a shrub, with trunks and branches in grey color with a wide layer of cork around them. The leaves are big, fleshy, and green in color (Figure 1). The flowers are purple, and its fruits are hollow, possessing only seeds with long fringes. It produces large quantities of latex in its green parts (Yaniv; Koltai, 2018). It is commonly distributed in coastal regions of Africa, Asia and South America, being considered exotic in Brazil. Recently, it is disseminated in arid and semiarid climates, being frequent in northeast Brazil, where it is known as “bombardeira” or “ciúmes” (Souto *et al.*, 2008).

Figure 1 – *Calotropis procera* in field before latex collection

Source: self-authorship

Different parts of the plant are used in alternative medicine. Roots, leaves, flowers, trunk and latex, have been utilized in aiding the treatment of various conditions, including pain, eczema and microbial infections (Amini *et al.*, 2021; Obese *et al.*, 2021). More importantly, the latex from this plant has been studied due to its effects over cellular immunity on various models. These effects include anti-inflammatory, proinflammatory and antimicrobial activity (Amaral *et al.*, 2021; Tavares *et al.*, 2021).

Among the constituents of *C. procera* latex, approximately 85 % of its mass is rubber. While soluble proteins represent around 10 %. The soluble protein fraction comprises proteins of molecular weight varying from 10 to 66 kDa. (Freitas, 2006).

Further investigations of LP showed that it contains chitinases (Freitas *et al.*, 2016), cysteine proteinases and osmotin (Freitas *et al.*, 2011), among many other unknown proteins. This protein fraction has been shown to control the immune system in various animal models, by modulating histamine, nitric oxide, and cytokines release, allowing anti-inflammatory and proinflammatory activity depending on the route of inoculation (Alencar *et al.*, 2006; Kumar; Sharma; Ramos, 2015).

1.3 The immune system

The immune system provides the most important barrier against pathogens and diseases. The immunological response against a novel pathogen

occurs in two levels, at a nonspecific range, mediated by the innate immunity, that acts by means of phagocytic cells and the complement system; and at specific range, mediated by the adaptive immunity, responsible for T-cell recognition and antibody production (Lewis; Williams; Eisenbarth, 2019).

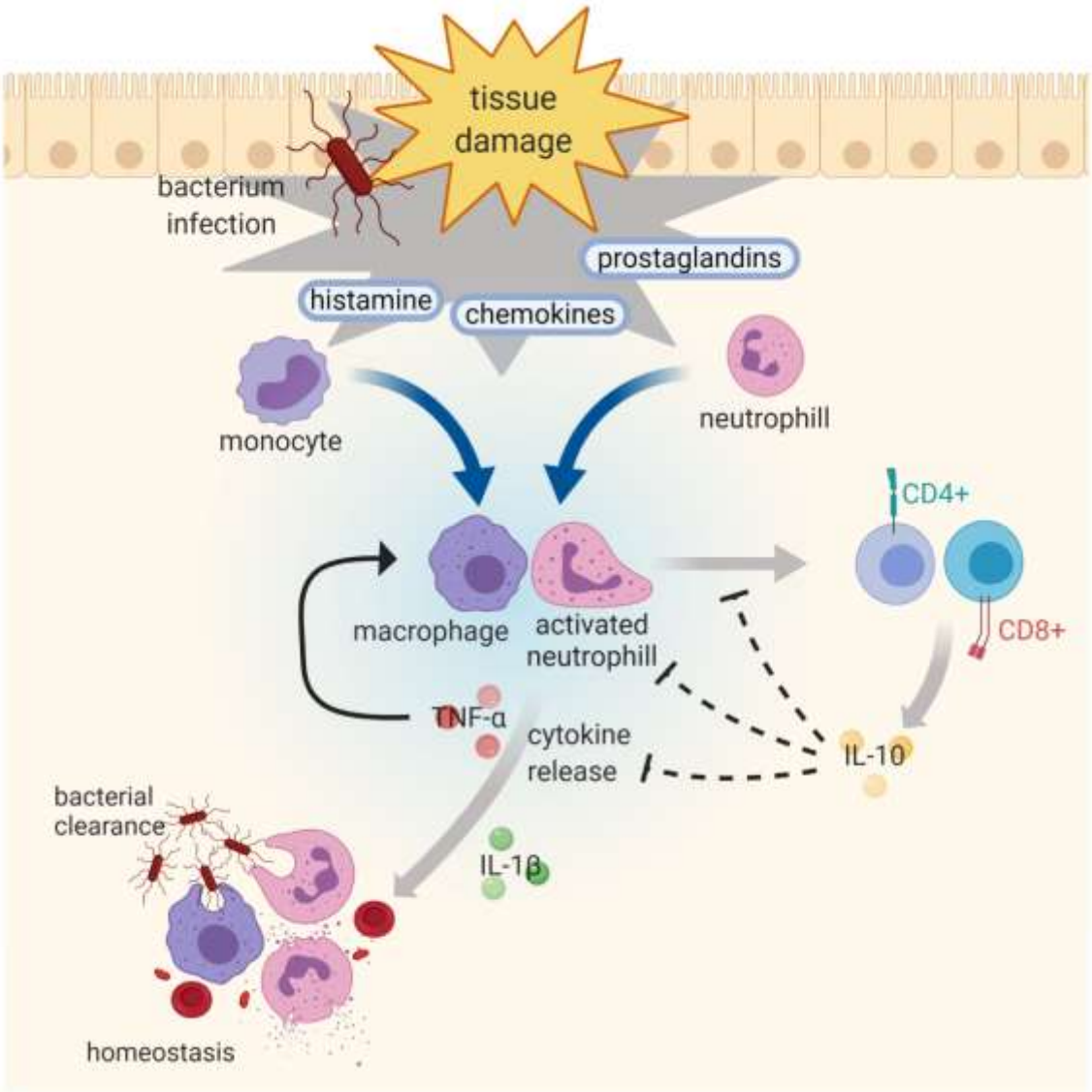
The key element of the immune response is the antigen presentation. It is executed by antigen presenting cells, which are usually dendritic cells and macrophages that possess pattern-recognition receptors strategically located within the cell. The antigen detected by these cells are molecular patterns associated with cell damage or with the pathogen. Once these patterns are recognized, these molecules activate the specific cell-mediated immunity and disseminate a systemic response (ANAYA et al., 2013).

Alongside the activation of cell-mediated immunity, there is the propagation of an inflammatory event, which is a nonspecific response to the entrance of a foreign body or pathogen into the organism, and it occurs in attempt to halt the dissemination of said infection (Figure 2). Once the previously described recognition occurs, macrophages and neutrophils are activated and start producing proinflammatory cytokines. Cytokines are peptides responsible for immunological signaling. The cell membrane is impermeable to these peptides, so they work through cell receptors, modulating the cell-based immune response that can act locally of systemically. Cytokines such as the tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) signal a system-wise reaction, including production and release of other cytokines, chemokines, and adhesion factors. Due to this signaling, the system starts to activate and recruit defensive cells that uses the adhesion molecules present in the endothelial lining to roll and traverse to the tissue to fight against the invader. This signaling also primes the organism to prevent pathogen dissemination by activating the complement system, coagulation cascade and altering vascular permeability (Poll *et al.*, 2017).

Another key mediator of the inflammation is the nitric oxide (NO) and other reactive oxygen species and are related to CYBB and NOX4 gene expression. They affect every step of the inflammatory process, low but fast increases in concentrations of NO, inhibit synthesis of cytokines, reduce leukocyte adhesion and the production of adhesion molecules, while large and lasting increases in concentrations of NO are toxic for the organism. The constitutive NO synthesis generates an anti-inflammatory effect, by inhibiting neutrophil activation; while

induced NO synthesis, generates proinflammatory response, activating neutrophils, causing apoptosis, and generating oxidative stress (Guzik; Korbut; Adamek-Guzik, 2003).

Figure 2 – The immune response



Source: self-authorship.

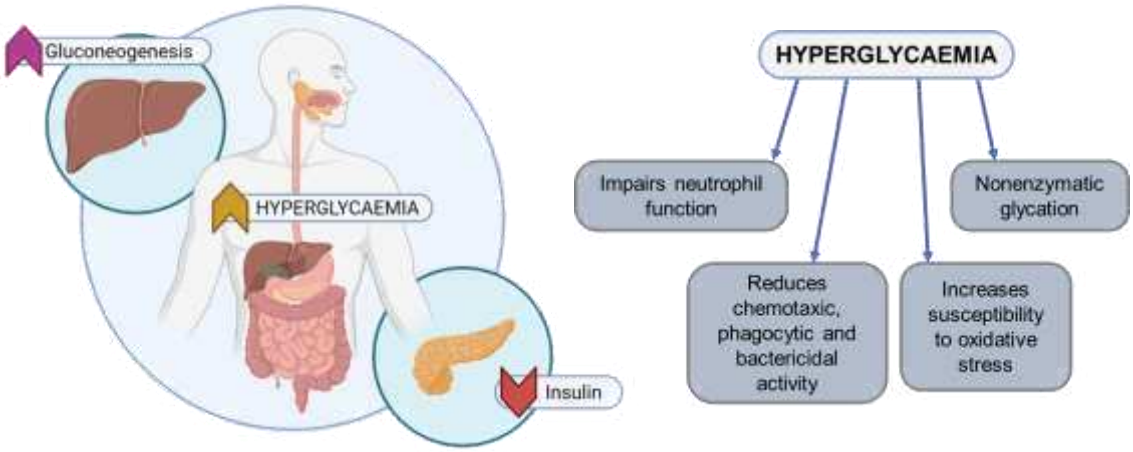
1.4 Pathophysiology of Diabetes Mellitus

Diabetes mellitus (DM) is a noncommunicable disease characterized by metabolic disorders, especially hyperglycemia and insensibility or resistance to insulin. In the long term, DM causes loss of structure and function of the pancreatic β-cells, while untreated diabetes usually lead to vascular complications and severe

immunological compromises. Currently, DM can be differently classified depending on its origin. (WHO, 2021; TONIOLO et al., 2019).

The elevated tolerance to insulin, or lack of its production, makes it difficult for glucose to enter cells and be metabolized, thus the liver starts compensating by increasing gluconeogenesis (Figure 3). This elevated hepatic glucose output is the main contributor to the increase in blood glucose levels (Zhang *et al.*, 2019).

Figure 3 – Pathophysiology of diabetes



Source: self-authorship

Hyperglycemia impairs neutrophil function, reducing its chemotaxis, phagocytic and bactericidal capacity. High glucose levels cause nonenzymatic glycation of various proteins, the increased glycation is associated with inhibited production of IL-10 and TNF-α. In insulin-independent tissues, the hyperglycemic environment increases the intracellular glucose levels, which require NADPH as cofactor to be metabolized. Decreased levels of NADPH reduce regeneration of antioxidant molecules, therefore increasing susceptibility to oxidative stress (Alves; Casqueiro; Casqueiro, 2012).

Elevated levels of blood glucose can increase blood viscosity; however, it is not the only factor to influence viscosity. Although some correlation has been found linking glycaemia to blood viscosity, it is understood that in DM patients, other associated risk factors, such as high blood pressure, elevated serum triglycerides, low HDL cholesterol levels, play a role in increasing BV (Mushtaq; Abdul Mateen; Kim, 2019). The micro-viscoelasticity of red blood cells (RBC) is also altered during

various diseases. Naturally, RBC suffer deformation when subjected to high shear forces, to flow through capillary vessels, for instance. The RBC from DM patients have been shown to be stiffer, requiring greater forces to deform, which might be linked to the vascular complications related to DM (Ciasca *et al*., 2015).

1.5 Pathogenesis of *Salmonella* Typhimurium

Salmonella enterica enterica serovar Typhimurium is a pathogenic bacterium that may cause typhoid-like disease in mice and acute gastroenteritis in humans. Although not usually fatal in humans, *Salmonella ssp*. induces fever, severe diarrhea and abdominal cholic. In humans, this bacterium is associated with enterocolitis, while in mice, it provokes symptoms like typhoid fever and it has been used as experimental models of inflammation and septic shock (Jong *et al*., 2012; Lima-Filho *et al*., 2004; To *et al*., 2021). *Salmonella* is also a good agent in models of infection for its ability to cause systemic infections from small inoculums, for being easily isolated from various organs and for being moderately resistant to antibiotics once inside monocytes, macrophages, neutrophils of its hosts (Portillo, 2001).

During infection, *Salmonella* attaches itself to the intestinal epithelia, its effector proteins modulate actin polymerization in the host enterocyte, resulting in integration of the bacteria with the target cell. When the *Salmonella* cells reaches the basal side of the epithelial cell, it is phagocyted by neutrophils and macrophages. Macrophages infected by *Salmonella* undergo apoptosis, releasing IL-1β that increases the inflammatory response. Neutrophils infected by *Salmonella* do not undergo apoptosis, the neutrophils recruited by the recently augmented inflammatory response, migrate through the epithelial layer, causing detachment of the basal membrane, fluid secretion into intestinal lumen and diarrhea. Proteases and inflammatory cells cause necrosis, facilitating bacterial growth and spread of the contamination (Santos *et al*., 2003).

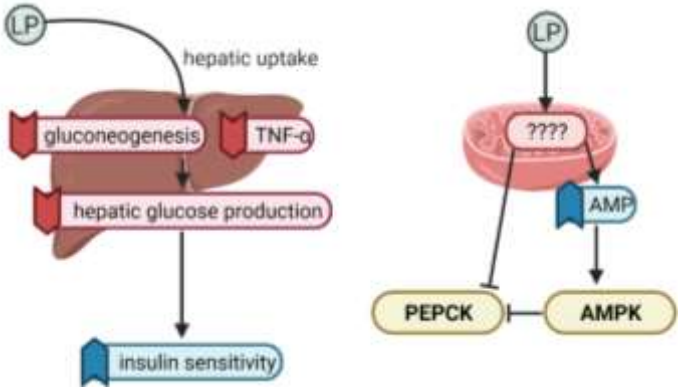
Bacterial infection may lead to septicemia, that stimulates the organism to activate genes coding for proinflammatory cytokines, such as TNF-α and IL-1β. If the advancement of the infection is not halted, a pleiotropic hyperinflammatory response can occur, leading to system-wise activation of the complement system, blood coagulation and alterations in vascular permeability. This systemic response leads to the septic shock, characterized by neutrophil dysfunction, apoptosis of lymphoid

cells, exacerbation of the inflammation and disseminated intravascular coagulation (Trivedi; Lalu, 2018-).

1.6 *Calotropis procera* latex in experimental models with metabolism and infection

Calotropis procera LP have been recently used in various experimental models to study its effect over different aspects of animal physiology. It was shown that LP, administered by intravenous route, reduced mouse glycemia, by activating AMPK pathway and reduction of PEPCK gene expression. It also ameliorated insulin and glucose tolerance. In DM patients, gluconeogenesis is a very active pathway, and its inhibition occurs similarly to the effect observed with LP, by activating AMPK pathway and suppressing PEPCK gene expression (Figure 4). These results show that LP has an effect on glycemic control and indicate therapeutic potential in treatment of Diabetes Mellitus (de Oliveira *et al.*, 2019).

Figure 4 – Effect of CpLP on the glucose metabolism



Source: self-authorship

The laticifer proteins from *Calotropis procera* were studied for their effect on bacterial infection of human interest. LP did not inhibit growth of *S.* Typhimurium in vitro; thus, this fraction does not act directly on the bacterium. When mice were treated by different inoculation routes, the dose of 60 mg/ kg of LP intraperitoneally was able to protect these animals against the lethal *Salmonella*, whereas the administration by oral and subcutaneous routes did not protect the animals. The histopathological analysis of liver and spleen revealed necrosis and inflammatory infiltrate. Although the treatment with LP prevented the death of the animals, bacteria

was still present in liver and spleen, causing histological damage. The reduction in bacterial load in the bloodstream of treated animals controlled the bacterial spread in the organs. TNF-α levels were not different between treated and control animals, but cultured macrophages treated with LP and stimulated by LPS released less IL-1β (Lima-Filho *et al.*, 2010).

A subfraction of LP was tested against the same *Salmonella* infection model. Animals treated by intraperitoneal route survived the infection challenge. Similarly, to the LP treatment, there was a high bacterial clearance in the bloodstream. TNF-α production was detected early in treated animals, alongside a reduction in nitric oxide contents in the blood. Neutrophil infiltration into the peritoneal cavity was enhanced and it is concluded that this inflammatory stimulus, caused by the protein inoculation, increased phagocytosis, preventing early animal death (Oliveira *et al.*, 2012).

Another subfraction of LP was tested on the *Salmonella* model and showed a different contribution towards the protection afforded by LP on previous experiments. Similarly, with this subfraction the recruitment of inflammatory cells was crucial. However, an increment in IL-10 levels, an anti-inflammatory cytokine may have controlled the advancement of the inflammation, allowing enough time for leukocyte migration, thus phagocytosis, to control bacteria spread (Sousa *et al.*, 2020).

Summarizing these findings, LP is shown to be a protein fraction of great biotechnological interest due to its activity over the immune system and against acute inflammation caused by bacterial infection. It should be noted that LP is not a single molecule, but a group of protein molecules that may each have a specific biological effect. Although these fractions exhibit similar effects, like recruitment of inflammatory cells, their actions can be perceived as opposite, sometimes proinflammatory, others anti-inflammatory. The protection against the lethal infection observed with LP is result of the synergistic action of its components, that can have cooperative or antagonistic mechanisms.

2 OBJECTIVES

2.1 General Objective

Investigate the effect of *Calotropis procera* laticifer proteins (CpLP) over the immune system of animals with induced hyperglycemia, subjected to an acute inflammatory process caused by bacterial infection.

2.2 Specific Objectives

- To establish a hyperglycemia induction model in female Swiss mice.
- To establish a model of acute inflammation in hyperglycemic Swiss mice using *Salmonella* Typhimurium.
- To investigate the influence of CpLP treatment on the immune cell blood profile and its migration pattern to the site of inflammation.
- To assess histological damage to target organs affected by the infection and the effect of CpLP treatment on the extent of this damage.
- To analyze the immunomodulatory effect of CpLP treatment on inflammatory cytokines.
- To verify the influence of hyperglycemia and CpLP treatment on whole blood viscosity.

3 PART I: C*alotropis procera* LATEX PROTEINS ENHANCE SURVIVAL AND REGULATE CYTOKINE RESPONSE IN HYPERGLYCEMIC SEPSIS

3.1 Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by hyperglycemia and impaired carbohydrate, lipid, and protein metabolism, resulting from defects in insulin secretion, action, or both (Alam *et al.*, 2021). DM affects more than 400 million people worldwide and is associated with increased morbidity and mortality due to its complications, such as cardiovascular diseases, nephropathy, retinopathy, and neuropathy (Akın; Bölük, 2020). The current pharmacological treatments for DM include insulin therapy and oral antidiabetic drugs, such as sulfonylureas, biguanides, thiazolidinediones, and alpha-glucosidase inhibitors. These drugs have limitations, such as adverse effects, loss of efficacy over time, and high cost. Therefore, there is a need for alternative and complementary therapies for DM, especially from natural resources, such as plants (American Diabetes Association, 2020; Blahova *et al.*, 2021).

Plants have been used for centuries as traditional remedies for various diseases, including DM. Bioactive compounds derived from plants, such as terpenes, flavonoids, alkaloids, and proteins, have been shown to exert antidiabetic effects through different mechanisms, such as stimulating insulin secretion, enhancing glucose uptake, inhibiting glucose absorption, modulating carbohydrate metabolism, and reducing oxidative stress (Alam *et al.*, 2022; Tran; Pham; Le, 2020).

Among the plants with antidiabetic potential, *Calotropis procera* (Aiton) W.T. Aiton, a member of the Apocynaceae family, has attracted considerable attention in recent years. Commonly known as Apple of Sodom, *C. procera* is a small tree native to arid and semi-arid regions of Africa and Asia (Yaniv; Koltai, 2018). It is widely used in folk medicine for the treatment of various ailments, such as skin infections, wounds, ulcers, fever, malaria, rheumatism, asthma, and cancer. The plant produces a milky latex that contains a complex mixture of proteins, lipids, carbohydrate, and secondary metabolites, such as cardenolides, flavonoids, and alkaloids. The latex has been reported to have various biological activities, such as antifungal, antibacterial, antiviral, antitumor, anti-inflammatory, and immunomodulatory effects (Amini *et al.*, 2021; Obese *et al.*, 2021). The total latex

can be toxic and irritant, and cause severe dermatitis, conjunctivitis and even blindness, but a lot of most recent studies suggest its protein fraction may have various biological activities with virtually no toxic effects.

Calotropis procera latex proteins (CpLP) when administered by intravenous route, reduced mouse glycemia, by activating AMPK pathway and reducing PEPCK gene expression. It also ameliorated insulin and glucose tolerance. In DM patients, gluconeogenesis is a very active pathway, and its inhibition occurs similarly to the effect observed with CpLP, by activating AMPK pathway and suppressing PEPCK gene expression. These results show that CpLP influences glycemic control and indicates therapeutic potential in treatment of DM (de Oliveira *et al.*, 2019). The latex protein fractions were studied for their effect on bacterial infection of human interest. *Calotropis procera* latex proteins did not inhibit growth of *Salmonella enterica* Typhimurium in vitro; thus, this fraction does not act directly on the bacterium. In animal models, the dose of 60 mg/ kg of CpLP intraperitoneally (*ip.*) was able to protect these animals against the lethal *Salmonella*, whereas the administration by oral and subcutaneous routes caused no protection (Lima-Filho *et al.*, 2010).

A subfraction of CpLP was tested against the same *Salmonella* infection model. Animals treated *ip.* survived the infection challenge. Neutrophil infiltration into the peritoneal cavity was enhanced and it is concluded that this inflammatory stimulus, caused by the protein inoculation, increased phagocytosis, preventing early animal death. Pro-inflammatory cytokines levels were increased by the treatment (Oliveira *et al.*, 2012). However, this subfraction also caused an increase in IL-10 levels, an anti-inflammatory cytokine may have systemically controlled the advancement of the inflammation, allowing enough time for leukocyte migration, thus phagocytosis, to control bacteria spread (Sousa *et al.*, 2020).

These studies show evidence of the immunomodulatory properties of *C. procera* latex and shine light onto possible therapeutic use of latex proteins in treatment of diabetes. Furthermore, the safety and efficacy of the latex extract in animals have been established. Via this research we aim to study the capabilities of CpLP in modulating the acute immune response on an organism immunosuppressed by diabetes.

3.2 Materials and methods

3.2.1 Animals

Swiss mice (n = 300, 21 ± 2 g) were obtained from the Universidade Federal do Ceará Central Animal House. All animals were specific pathogen free (SPF). All animal experiments were carried out in accordance with the Brazilian National Council of Control of Animal Experimentation (CONCEA). The animal experimentation was approved by Animal Care and Use Committee (CEUA) of the Universidade Federal do Ceará under the protocol No.: 2742280720.

3.2.2 Microorganism

The systemic infection of mice was provoked with *Salmonella enterica enterica* serovar Typhimurium C5 strain. The bacteria were activated in Brain Heart Infusion (BHI) broth at 37 ºC for 24 hours and then cultured in BHI agar for another 24 hours at 37 ºC. For the infection experiments, bacteria colonies were taken from the BHI agar plates, suspended in Phosphate Buffered Saline (PBS) until an optical density at 600 nm of 0.5 was reached. This suspension, containing 10^8 Colony Forming Units per mL (CFU/ mL) was further diluted into the working concentrations mentioned below.

3.2.3 Latex processing

After identifying *C. procera* plants in the field, the latex was collected by breaking each shoot, the exuded latex was collected in tubes containing distilled water (1:1, v/v), which were centrifuged at 10,000 x g (4 ºC for 10 minutes). The supernatant, mostly free of rubber, was dialyzed against distilled water for 60 hours at 4 ºC, using dialysis membranes with 8 kDa of cutoff size. The sample was centrifuged again under the same conditions, and the supernatant, devoid of rubber and rich in proteins, was lyophilized, to obtain soluble proteins, which were called CpLP. (Alencar *et al.*, 2006).

3.2.4 Experimental design

To investigate the effects of CpLP over the immune system of diabetic animals, diabetes was induced using two different protocols with Streptozotocin (STZ). The first protocol used a single high dose of STZ (180 mg/ kg), and the second protocol used 5 consecutive low doses (5x40 mg/ kg). Both protocols were adapted from a well-known protocol for chemical diabetes induction (Furman, 2021). The peripheral blood glycemia of the animals was measured every two days using a portable glycosometer. All hyperglycemic animals (blood glucose greater than 220 mg/ dL) were divided into different groups. The most efficient protocol for diabetes induction was chosen, based on hyperglycemia intensity, animal well-being and survivability.

To determine the ideal bacterial concentration, for an acute inflammation assay, three different concentrations of *S.* Typhimurium suspension were tested on diabetic animals (10^2, 10^4, 10^6 CFU/ mL). The ideal concentration was found as the lowest lethal dose to cause 100 % of death to animals within 3 to 5 days after infection, in order to provide a good time window for further investigation (Lima-Filho et al., 2010).

For investigating the protective effect of CpLP treatment over the immune system while under acute inflammation, diabetic animals were treated with either 30 or 60 mg/ kg of CpLP, 24 hours prior to being infected with the lethal dose of *S.* Typhimurium, found in the previous experiments.

Peripheral blood glycemia was measured during diabetes induction, and prior to experimentation with infection. After 24 and 48 hours of infection, all animals were euthanized and their blood, peritoneal fluid, liver, spleen, kidney and pancreas were collected for further analysis.

3.2.5 Measurement of peripheral blood glucose

The measurement of glycemia was done by measuring capillary glucose on the tail of mice. A drop of blood was drawn by puncture with a sterile needle, and a blood glucose meter was used to measure glucose levels. After measuring, the puncturing wound was pressed with sterile gauze until the bleeding stopped. For repeated measurements on the same animals, a new puncture was avoided by

rubbing the scarred tissue in gauze, until the bleed starts again. Blood glycemia levels were expressed as mg/ dL.

3.2.6 Blood and peritoneal fluid processing

For the investigation of hematological parameters and cytokines, the blood was drawn from the retroorbital plexus with a glass capillary tube in anesthetized animals. For the collection of peritoneal fluid (PF), euthanized animals had their abdominal cavity filled with sterile PBS, following an abdominal massage, the fluid was collected. Blood and PF were collected in EDTA tubes. Blood vials were analyzed by a semi-automatic hematological analyzer, looking for: white cell, lymphocytes, granulocytes, red blood cells count; hemoglobin, hematocrit, and platelets. The remaining blood and PF were directed to further analysis.

3.2.7 Histopathology of organs and adipose tissue

The abdominal cavity was opened, liver, spleen, kidneys, pancreas were harvested, taking necessary precautions to not damage the organs during the procedure.

To evaluate possible organ damage associated with the inflammatory process and the effect of diabetes, the organs were fixed, dehydrated, diaphanized and included in paraffin for micro cutting into 5 µm slices. The slices were mounted on microscope slides and stained with hematoxylin and eosin. The slides were analyzed by a blind, unbiased pathologist, in search for the presence of inflammatory infiltrate, structural damage or any abnormal event.

3.2.8 Cytokine measurement

The levels of TNF-α, IL-1β and IL-10 were measured in the blood plasma and peritoneal fluid. The samples were centrifuged to obtain plasma. ELISA kits for the respective cytokines were strictly followed (R&D Systems). The results were expressed as ρg of cytokine per mL of fluid.

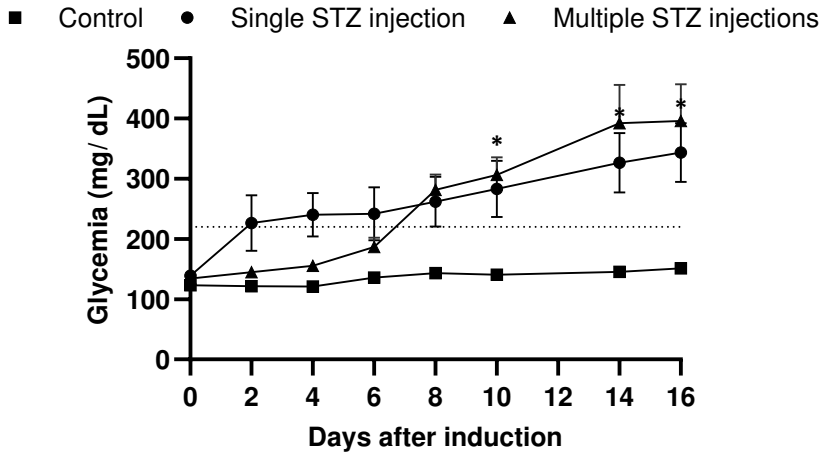
3.2.9 Statistical analysis

All data were expressed as mean ± standard error of the mean and were analyzed using GraphPad Prism 10. Outliers were detected and removed using ROUT test. Comparison among groups were performed using One-way analysis of variance (ANOVA) with Tukey post-hoc. Statistical significance was considered when values of $p < 0.05$.

3.3 Results

Hyperglycemia was successfully induced in mice with both protocols. Average peripheral blood glycemia was higher than 220 mg/ dL 2 days after induction with single STZ injection, and after 10 days, more than 70 % of animals showed stable levels of blood sugar higher than the selected threshold (Figure 5). Multiple STZ injection also showed stable levels of blood glucose after 10 days, with high induction rate (>70 %), but the glycemic increase was slower than with a single STZ injection. Increased urination and water consumption were not recorded but were noted after induction. The caveat of the repeated doses protocol was that the consecutive injections raise the animal's distress and provoke multiple edemas on the site of injection on the abdomen. Due to the scope of these experiments, their execution was split between multiple researchers. It was not possible to ensure the same quality of animal handling between the researchers. For these multiple reasons, but especially due to the animal's well-being, the protocol chosen for the remaining of the experiments was the single STZ injection.

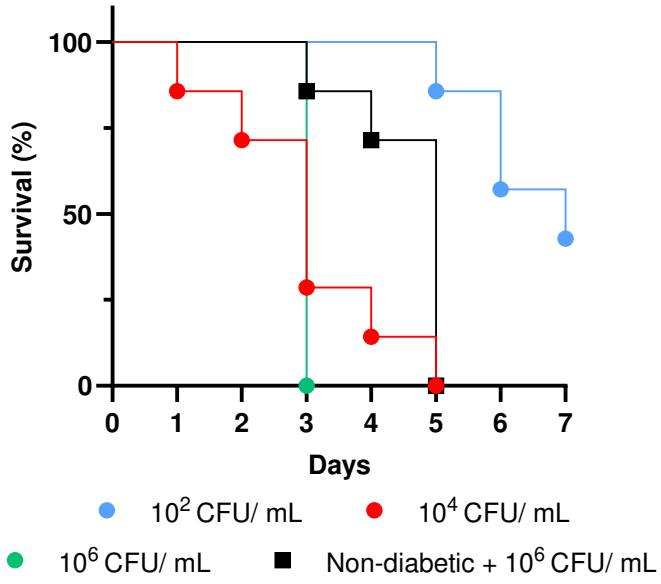
Figure 5 - Peripheral blood glycemia mice subjected to different diabetes induction.



Source: self-authorship.
After induction with a single high dose of STZ (180 mg/ kg), and multiple low doses of STZ (, blood glycemia was measured at the tail of each mice every 2 days. Mice were considered diabetic when blood glucose reached 220 mg/ dL or higher (dotted horizontal line). In this study, within 10 days post induction, glycemia levels of induced animals were significantly different than that of healthy animals. Some error bars cannot be shown because they are shorter than the symbols. * $p < 0.05$ versus non-diabetic group ($n = 5$, Two-Way ANOVA, Bonferroni).

A model of acute inflammation was employed using a lethal infection with *Salmonella* as the inductor of the acute inflammation. To design for the best time window of investigation, a lethal dose was found as the dosage of bacteria that would cause death to all the animals within 3 to 5 days of infection. In these experiments hyperglycemic animals were more susceptible to death from the infection. The concentration of 10^4 CFU/ mL met the requirement of mortality for this study (Figure 6).

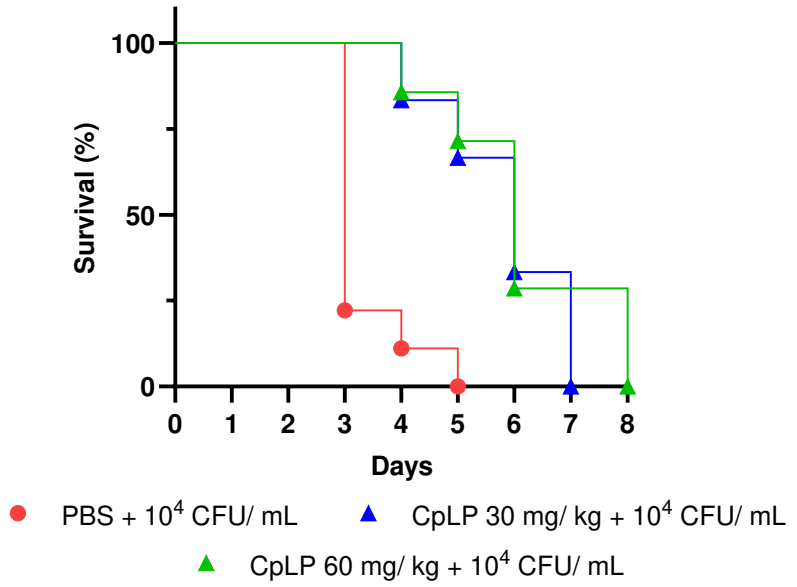
Figure 6 - Infection challenge survival of hyperglycemic mice.



Source: self-authorship.
Diabetic mice were challenged with three different concentrations of *S.* Typhimurium suspension (10^2, 10^4, 10^6 CFU/ mL). The survival percentage of each infected group was measured within 7 days from infection. A non-diabetic control group was tried with 10^6 CFU/ mL. Diabetic animals showed more susceptibility to infection and higher death rate. All curves are significantly different (n = 10, Log-rank (Mantel-Cox) test).

Having chosen the bacterial concentration, animals were treated ip. with either two concentrations of CpLP (30 or 60 mg/ kg), 24 hours prior to the infection with 10^4 CFU/ mL of *Salmonella*. The treatment extended the life of infected animals for 2 days, when compared to untreated animals, but ultimately, they all died (Figure 7). Although animals treated with 60 mg/ kg survived one day longer, there was no statistical difference between the survival rate of both treated groups. For this reason, and to save resources, the concentration of 30 mg/ kg was chosen for the rest of the investigation.

Figure 7 - Protection challenge survival of hyperglycemic mice.



Source: self-authorship
Diabetic mice were treated with two concentrations of CpLP (30, 60 mg/ kg) and challenged with 10^4 CFU/ mL of *S.* Typhimurium. The survival percentage of each infected group was recorded until all animals died. A control group whose animals received PBS instead of CpLP was included. Treated animals showed higher survival than non-treated ones.
All curves are significantly different (n = 10, Log-rank (Mantel-Cox) test).

Hematological examination revealed that CpLP increased the numbers of lymphocytes and polymorphonuclear cells, i.e.: neutrophils. Infection caused an increase in total number of white blood cells but caused reduction in ratio of lymphocytes (leukopenia), while of levels polymorphonuclear cells remained elevated (Table 1).

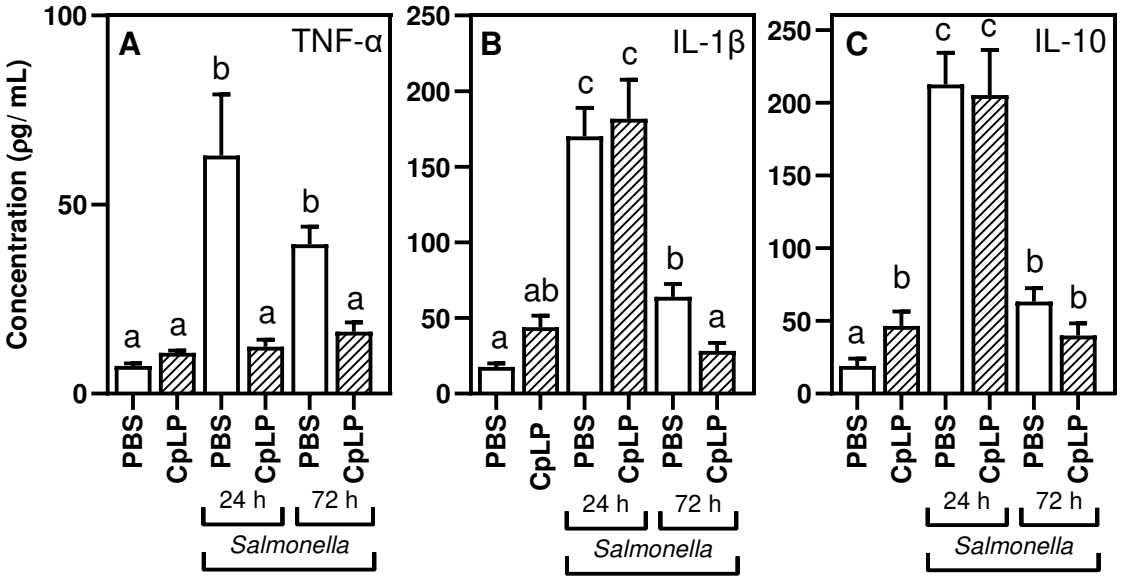
Table 1 - Hematologic profile hyperglycemic mice treated with CpLP and infected *Salmonella*.

	PBS	**CpLP**	**CpLP + *Sal*. 24 h**	**CpLP + *Sal*. 72 h**
RBC (x10^6 cells/ µL)	9.78 ± 0.27	9.80 ± 0.26	9.89 ± 0.16	9.62 ± 0.19
HB (g/ L)	152.33 ± 3.18	147.50 ± 5.19	152.40 ± 2.32	148.75 ± 1.31
HC (%)	46.67 ± 0.88	45.25 ± 1.55	48.20 ± 0.58	45.50 ± 0.29
WBC (x10^3 cells/ µL)	2.73 ± 0.18	3.33 ± 0.10	4.47 ± 0.14*	5.43 ± 0.39*#
PMN (%)	20.00 ± 0.94	41.00 ± 6.26*	46.67 ± 2.58*	39.67 ± 3.82*
LYM (%)	66.00 ± 4.50	69.33 ± 2.93*	51.25 ± 2.49*#	39.00 ± 1.80*#
EOS (%)	1.33 ± 0.67	1.00 ± 0.41	1.40 ± 0.24	0.50 ± 0.29
MON (%)	4.67 ± 1.45	4.50 ± 1.89	6.40 ± 1.17	9.25 ± 3.68
PLA (x10^3 cells/ µL)	630.67 ± 30.14	623.00 ± 32.11	970.25 ± 37.08	698.67 ± 31.07
PRO (g/ dL)	4.53 ± 0.29	4.75 ± 0.29	5.12 ± 0.12	4.40 ± 0.18

Source: self-authorship
RBC: red blood cells. HB: hemoglobin. HC: hematocrit. WBC: white blood cells. PMN: polymorphonuclear. LYM: lymphocytes. EOS: eosinophils. BAS: basophils. MON: monocytes. PLA: platelets. PRO: protein.
*: $p < 0.01$ against PBS.
#: $p < 0.01$ against CpLP.
(n = 5, One-way ANOVA).

To assess the cytokine response in the peritoneal fluid (Figure 8), we measured TNF-α, IL-1β and IL-10 levels at 24 and 72 hours after infection. We found that infection alone induced a sharp rise in TNF-α within 24 hours, followed by a decline over 72 hours. CpLP treatment did not affect TNF-α by itself, but it prevented the infection-induced elevation and kept it at normal levels for 72 hours. IL-1β levels also increased after infection, but they peaked at 24 hours and then decreased, although they remained higher than baseline. CpLP treatment only influenced IL-1β after 72 hours, restoring it to the initial level. IL-10 levels were elevated by infection and stayed high for 72 hours. CpLP treatment increased IL-10 before infection but did not alter its course during infection.

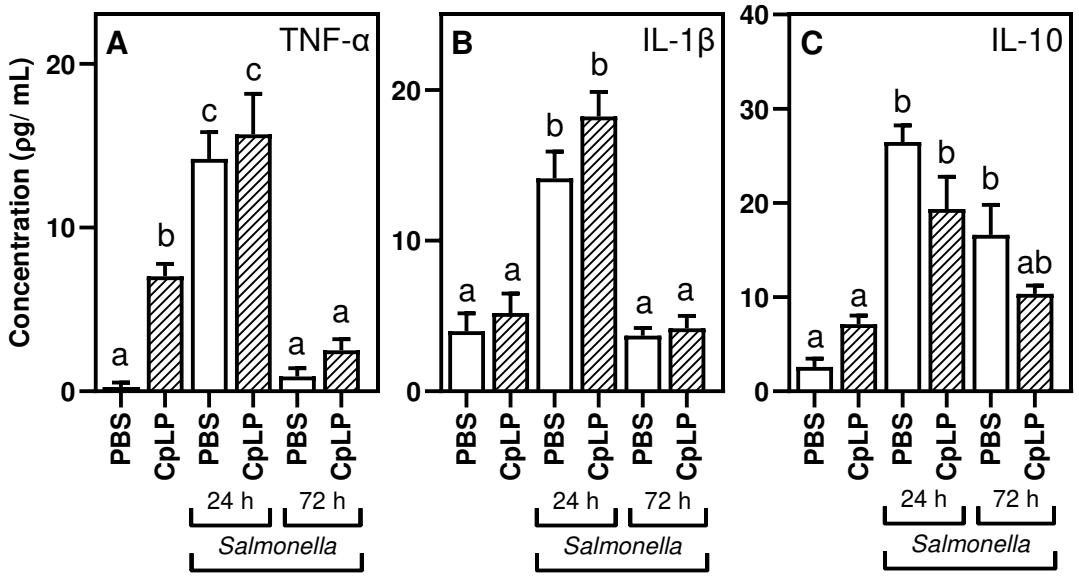
Figure 8 - Cytokine measurement of the peritoneal fluid of hyperglycemic mice treated with CpLP and challenged *Salmonella*



Source: self-authorship
Animals were euthanized 24 and 72 hours after infection, peritoneal cavities were washed, and fluid was collected and processed for cytokine measurement using ELISA kits.
Different letters indicate statistically significant differences (n = 5, One-way ANOVA).

In the blood plasma, we observed a transient increase in TNF-α and IL-1β after infection, which returned to normal after 72 hours. CpLP treatment increased TNF-α in the plasma, but it also normalized after 72 hours. CpLP treatment had no effect on IL-1β or IL-10 in the plasma, which followed the same pattern as the infected-untreated group (Figure 9).

Figure 9 – Cytokine measurement of the blood plasma of hyperglycemic mice treated with CpLP and challenged *Salmonella.*

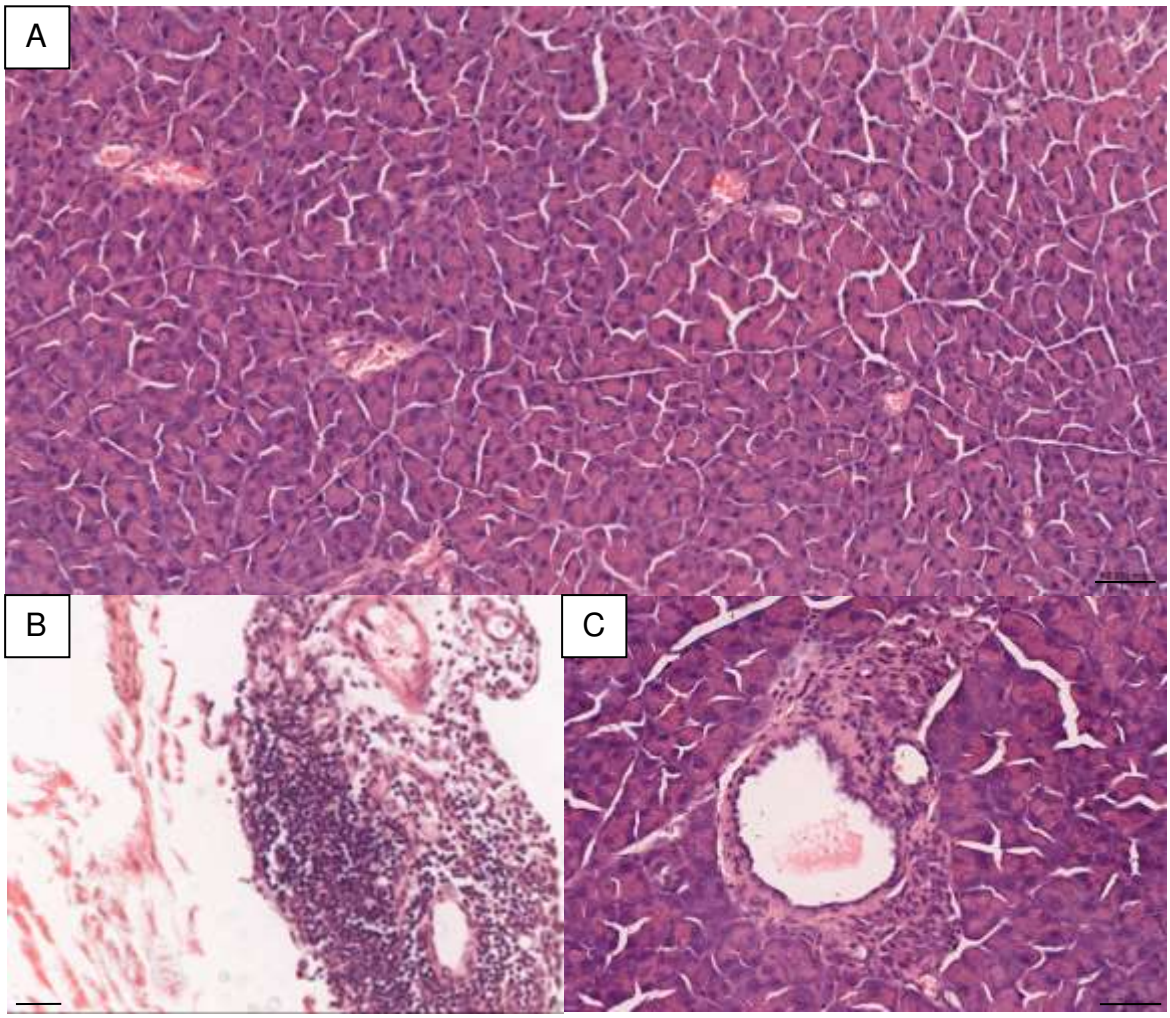


Source: self-authorship.

Animals were euthanized 24 and 72 hours after infection, blood was collected and processed for plasma cytokine measurement using ELISA kits.
Different letters indicate statistically significant differences (n = 5, One-way ANOVA, $p<0.05$).

The histopathological analysis revealed the damage Streptozotocin caused to the pancreas of all animals who underwent diabetes induction. There was a significant reduction in pancreatic islet cells (Figure 10 A), accompanied by strong lymphocyte infiltration into the peripancreatic fat region (Figure 10 B). The treatment with CpLP intensified the inflammatory infiltration, which became present even in the periductal region (Figure 10 C). There was no other histological finding in the pancreas of the other groups.

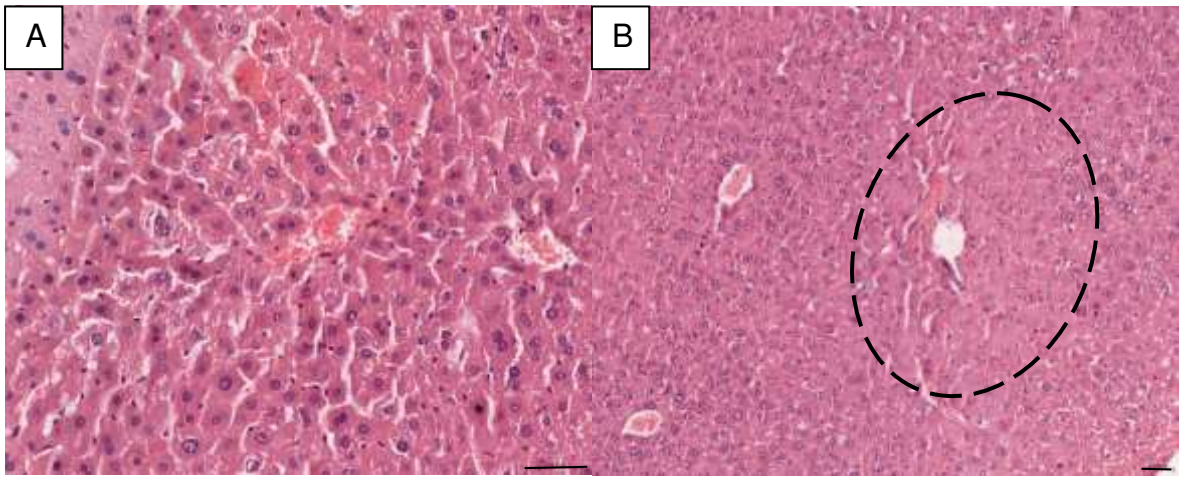
Figure 10 – Histopathological analysis of the pancreas of animals who underwent diabetes induction, treated with CpLP and infected with *Salmonella.*



Source: self-authorship.
Microscopic slides of pancreas of animals who underwent diabetes induction. All hyperglycemic animals had significant reduction in pancreatic islets as seen in A, and a significant lymphocytic infiltration into the peripancreatic fat, as seen in B. All groups that received CpLP had significant lymphocytic infiltration in the periductal region, as seen in C.
The black bar in the corner of the images represents 0.05 mm.

The treatment with CpLP caused focal acute inflammation and Kupffer cell hyperplasia in the liver. The infection alone caused tissue degeneration, which was exacerbated after 72 hours of infection (Figure 11 A). The treatment prior to infection showed signs of generalized inflammation after 24 hours of infection and focal necrosis after 72 hours (Figure 11 B).

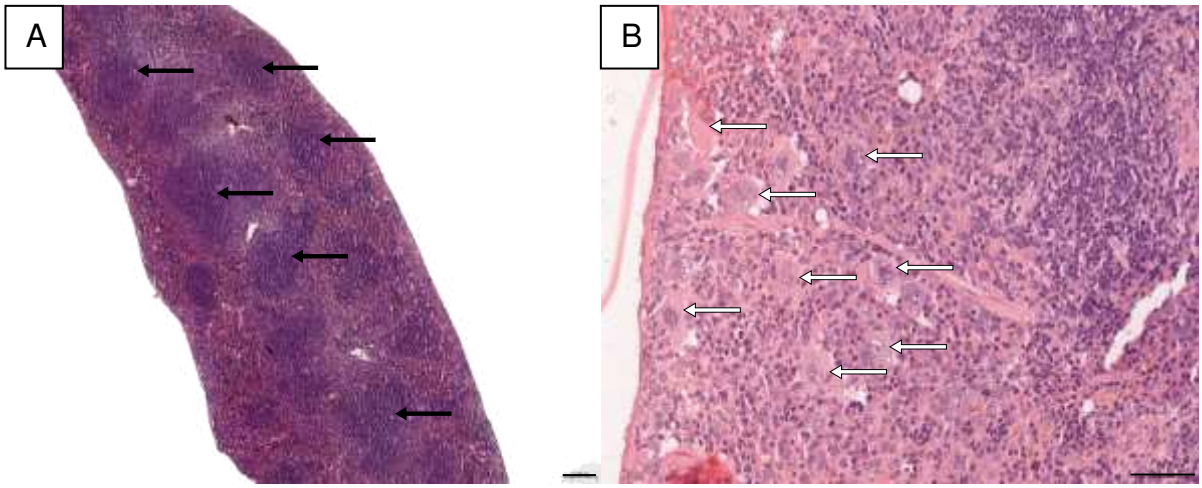
Figure 11 - Histopathological analysis of the liver of animals who underwent diabetes induction, treated with CpLP and infected with *Salmonella.*



Source: self-authorship.
Microscopic slides of liver of animals who underwent diabetes induction. Infection caused excessive tissue degeneration, which was exacerbated after 72 hours, as seen in A. The treatment with CpLP caused multiple focal necrosis, as seen circled in B.
The black bar in the corner of the images represents 0.05 mm.

The only histological findings in the spleen were lymphoid hyperplasia of the white pulp on all infected animals; and megakaryocytosis in all treated animals, infected or not (Figure 12).

Figure 12 - Histopathological analysis of the spleen of animals who underwent diabetes induction, treated with CpLP and infected with *Salmonella.*



Source: self-authorship.
Microscopic slides of liver of animals who underwent diabetes induction. Infection caused an increase in number of lymphocytes on the white pulp (black arrow), as seen in A. Treatment with CpLP Caused increased number of megakaryocytes in the spleen (white arrow), as seen in B.
The black bar in the corner of image A represents 0.2 mm, and in 0.05 mm in image B.

There were no histological findings in the kidneys.

Representative images of all organs and treatments are presented in Appendices A – D.

3.4 Discussions

We successfully induced hyperglycemia in mice by injecting them with streptozotocin, a drug that destroys pancreatic beta cells and impairs insulin secretion (Lenzen, 2008). The hyperglycemic mice showed stable levels of blood glucose above 220 mg/dL after 10 days of induction, which is consistent with previous studies (King, 2012; Szkudelski, 2001). We also observed increased urination and water consumption in the hyperglycemic mice, which are common signs of diabetes (Liu; Daneshgari, 2014). The mortality rate during induction was lower than 20 %, which is acceptable for this type of experiment (Szkudelski, 2001).

To study the immune response, we infected the hyperglycemic mice with a lethal dose of bacteria, which we determined as the concentration that would cause 100 % mortality within 96 hours of infection. We found that the hyperglycemic mice were more susceptible to the bacterial infection than the normoglycemic mice, as they died faster and in higher numbers. This result agrees with previous reports that diabetes increases the risk and severity of infections (Geerlings; Hoepelman, 1999; Peleg *et al.*, 2007). The concentration of 10^4 CFU/ mL met the requirement of mortality for this study, as it induced a rapid and fatal infection in both hyperglycemic and normoglycemic mice (Figure 6).

We then tested the effect of CpLP treatment on the survival and immune response of the infected mice. CpLP has been shown to have antibacterial, antifungal, antiviral, and immunomodulatory properties (Bezerra *et al.*, 2017; Kumar *et al.*, 2015, 2011). We administered two doses of CpLP (30 and 60 mg/kg) intraperitoneally to the mice 24 hours before the infection. We found that CpLP treatment extended the survival of the infected mice for 2 days, compared to the untreated mice, but they all eventually died (Figure 7). This suggests that CpLP treatment had a protective effect against the bacterial infection, but it was not sufficient to prevent death. The higher dose of CpLP (60 mg/kg) did not confer any additional benefit over the lower dose (30 mg/kg), as there was no significant difference in the survival rate between the two treated groups. Therefore, we chose the lower dose of CpLP for the rest of the investigation, as it was more economical and less likely to cause adverse effects.

To assess the cytokine response in the peritoneal fluid, we measured the levels of TNF-α, IL-1β, and IL-10 at 24 and 72 hours after infection. These cytokines

are important mediators of inflammation and immunity, and their dysregulation can lead to tissue damage and organ failure (Dinarello, 2000; Tracey, 2002). We found that infection alone induced a sharp rise in TNF-α within 24 hours, followed by a decline over 72 hours. TNF-α is a pro-inflammatory cytokine that plays a key role in the host defense against bacterial infections, but it can also cause systemic inflammation and shock if overproduced (Tracey, 2002; Van Der Poll; Opal, 2008). CpLP treatment did not affect TNF-α by itself, but it prevented the infection-induced elevation and kept it at normal levels for 72 hours. This result indicates that CpLP treatment modulated the TNF-α response and reduced the inflammatory damage caused by the infection.

IL-1β levels also increased after infection, but they peaked at 24 hours and then decreased, although they remained higher than baseline. IL-1β is another pro-inflammatory cytokine that enhances the immune response and induces fever, but it can also contribute to tissue injury and sepsis if uncontrolled (Dinarello, 2000; Van Der Poll; Opal, 2008). CpLP treatment only influenced IL-1β after 72 hours, restoring it to the initial level. This result suggests that CpLP treatment had a delayed effect on IL-1β and attenuated the prolonged inflammation caused by the infection.

IL-10 levels were elevated by infection and stayed high for 72 hours. IL-10 is an anti-inflammatory cytokine that suppresses the production of pro-inflammatory cytokines and limits the inflammatory response, but it can also impair the clearance of pathogens and increase the susceptibility to infections (Moore et al., 2001; O'Garra & Vieira, 2007). CpLP treatment increased IL-10 before infection but did not alter its course during infection. This result implies that CpLP treatment enhanced the anti-inflammatory response and prevented excessive inflammation, but it did not compromise the host defense against the infection.

In the blood plasma, we observed a transient increase in TNF-α and IL-1β after infection, which returned to normal after 72 hours. This result reflects the systemic inflammatory response to the infection, which can lead to multiple organ dysfunction and death (van der Poll & Opal, 2008; Angus & van der Poll, 2013). CpLP treatment increased TNF-α in the plasma, but it also normalized after 72 hours. This result may indicate that CpLP treatment stimulated the systemic immune response and enhanced the resistance to the infection, but it did not cause a sustained inflammatory reaction. CpLP treatment had no effect on IL-1β or IL-10 in the plasma, which followed the same pattern as the infected-untreated group. This

result suggests that CpLP treatment did not interfere with the systemic regulation of these cytokines.

The histopathological analysis revealed the mechanism by which Streptozotocin induces hyperglycemia in diabetes models. STZ is a compound that is particularly toxic to the insulin-producing beta cells of the pancreas in mammals. It is used to induce diabetes in animal models due to its ability to destroy beta cells. The mechanism by which STZ causes beta cell death involves multiple steps. STZ is structurally similar to glucose, which allows it to be transported into the beta cells via the GLUT2 glucose transporter, a protein that beta cells use to sense blood glucose levels. Once inside the beta cells, STZ causes damage by alkylating DNA. This occurs through its methylnitrosourea moiety, which can modify biological macromolecules, leading to DNA fragmentation. The DNA damage induced by STZ activates poly(ADP-ribose)polymerase (PARP), an enzyme involved in DNA repair. However, excessive activation of PARP can deplete cellular stores of nicotinamide adenine dinucleotide (NAD), leading to cell death. The culmination of DNA damage, PARP activation, and NAD depletion results in the death of beta cells. This process mimics the loss of beta cell function seen in type 1 diabetes (Furman, 2021).

It's important to note that while STZ is effective for inducing diabetes in rodent models, human beta cells have shown resistance to STZ-induced death, suggesting species-specific differences in beta cell vulnerability to this compound (Yang; Wright, 2002). These mechanisms highlight the complexity of STZ's action and its utility in diabetes research, providing a means to study the disease and potential treatments. However, the use of STZ also underscores the need for careful consideration of its effects and the relevance of animal models to human diabetes.

The intensification of inflammatory infiltration with CpLP treatment, particularly in the periductal region, suggests an amplified immune response, which may be both beneficial and detrimental. This dual effect of CpLP treatment is reminiscent of various past findings, who reported similar inflammatory responses with immunomodulatory treatments. One study explored the effects of a sub-fraction of CpLP (LP-PI) on the inflammatory response in Swiss mice challenged by *Salmonella* Typhimurium (Oliveira *et al.*, 2012). Another study highlighted the phytomodulatory activity of the purified protein fraction from the latex against various clinically relevant inflammatory conditions, such as arthritis, cancer, and sepsis

(Ramos *et al.*, 2020). Additionally, a systematic review on the biological evaluation of *Calotropis procera* emphasized the anti-inflammatory effects observed in the latex but also mentioned that excessive intake could have negative health impacts (Dogara, 2023). These studies suggest that the proteins from the latex of *Calotropis procera* may play a significant role in modulating the immune response, yet they also underscore the need for caution due to potential adverse effects.

Focal acute inflammation and Kupffer cell hyperplasia suggest that the treatment activates the liver's resident macrophages, which are crucial for clearing pathogens and debris but can also contribute to inflammation when overstimulated. The generalized inflammation and focal necrosis observed post-treatment indicate that while *Calotropis procera* may have therapeutic potential due to its anti-inflammatory properties, it can also lead to an overactive immune response (Patel *et al.*, 2021). This overactivation can result in tissue damage beyond what is caused by the infection alone, as the body's attempt to heal can sometimes become dysregulated and destructive. It's a delicate balance between fighting the infection and preventing the immune system from harming the body's own tissues. These findings underscore the importance of dosage and context in the use of *Calotropis procera* for medicinal purposes, as well as the need for further research to optimize its use and minimize potential adverse effects. It's also a reminder of the complexity of immune responses and the challenges in harnessing them for therapeutic benefit without causing harm.

Infection with *Salmonella* can lead to changes in the splenic architecture, including the effacement of splenic architecture and drastic changes in cell proportions and their in situ distribution. Most notably, red blood cells and red pulp macrophages expand beyond the red pulp and "take over" white pulp areas, thus contributing to the redistribution of lymphocytes in the spleen (Rosche et al., 2015). Localized lymphoid hyperplasia of the spleen, as seen in this study (Figure 9), a rare benign condition, typically entails a diffuse expansion of white pulp throughout the spleen. The etiology of localized lymphoid hyperplasia is unknown, though it is speculated to represent a localized response to antigenic stimulation akin to florid reactive hyperplasia in a solitary lymph node (Muir; Espinas; Delabie, 2016).

Interestingly, no histological changes were observed in the kidneys, indicating organ-specific responses to CpLP treatment and *Salmonella* infection. This organ specificity has been noted in other studies, emphasizing the need for targeted

therapies. This specificity is likely due to the complex interactions between the bioactive compounds in the latex and the unique physiological environments of different organs. The absence of histological changes in the kidneys in this context suggests that the kidneys may either be less responsive to the inflammatory and immune-modulating effects of the latex proteins or that the mechanisms of action of these proteins do not significantly impact renal tissue.

It's important for further research to explore these organ-specific effects more deeply to understand the full therapeutic potential and limitations of *Calotropis procera* latex proteins. This knowledge could help tailor treatments for specific conditions while avoiding unintended effects on other organs.

In summary, we demonstrated that CpLP treatment improved the survival and modulated the cytokine response of hyperglycemic mice infected with bacteria. CpLP treatment prevented the infection-induced elevation of TNF-α and IL-1β in the peritoneal fluid and restored them to normal levels after 72 hours. CpLP treatment also increased IL-10 before infection and maintained it at high levels during infection. CpLP treatment had a similar effect on TNF-α in the blood plasma, but it did not affect IL-1β or IL-10 in the plasma. These results suggest that CpLP treatment balanced the pro-inflammatory and anti-inflammatory responses and reduced the inflammatory damage caused by the infection, but it did not compromise the host defense against the infection. However, CpLP treatment was not able to prevent death in the infected mice, indicating that the bacterial infection was too severe and overwhelming for the hyperglycemic mice. Further studies are needed to optimize the dose and timing of CpLP treatment and to explore its mechanisms of action and potential applications in the treatment of infections in diabetic patients.

3.5 Conclusion

Here it was elucidated the complex interplay between hyperglycemia, bacterial infection, and CpLP treatment in a murine model. Our research demonstrated that CpLP treatment significantly modulates the cytokine response, improving survival rates and mitigating inflammatory damage without compromising the host's defense mechanisms against infection. Notably, the treatment normalized the levels of pro-inflammatory cytokines TNF-α and IL-1β in the peritoneal fluid and maintained elevated levels of the anti-inflammatory cytokine IL-10, suggesting a therapeutic balancing of immune responses.

Histopathological findings revealed the detrimental impact of Streptozotocin on pancreatic islet cells, with CpLP treatment intensifying inflammatory infiltration, indicating an amplified immune response. In the liver, CpLP treatment induced acute inflammation and Kupffer cell hyperplasia, while the infection alone led to tissue degeneration, exacerbated by the treatment. The spleen showed lymphoid hyperplasia and megakaryocytosis across all infected and treated animals, highlighting the systemic nature of the immune response.

However, the treatment's inability to prevent mortality in the face of severe bacterial infection underscores the need for further optimization of CpLP's dosage and timing. Moreover, the absence of histological changes in the kidneys suggests an organ-specific response to both the disease and treatment, warranting additional investigation.

In conclusion, our findings contribute to the understanding of the pathophysiological mechanisms in diabetic infections and offer a foundation for future studies aimed at improving therapeutic strategies. While CpLP holds promise, its full potential can only be realized through continued research and clinical trials to refine its application in managing infections among diabetic populations.

4 PART II: BLOOD RHEOLOGY IN HYPERGLYCEMIA: EFFECT OF BACTERIAL INFECTION AND MODULATION BY PLANT LATEX PROTEINS

4.1 Introduction

Diabetes mellitus (DM), a prevalent chronic metabolic disorder, significantly impacts global health by predisposing millions to cardiovascular complications (Alam *et al.*, 2021). A critical factor in the onset and advancement of these complications is blood rheology – essentially the study of how blood flows and deforms under various conditions (Akın; Bölük, 2020). Blood viscosity, indicating the flow resistance of blood, serves as a crucial rheological parameter, encapsulating the dynamic interactions among blood cells and plasma components. This complexity renders blood a non-Newtonian fluid, exhibiting unique flow behavior that deviates from the linear viscosity seen in Newtonian fluids. The Bingham model (Verma, 2014) stands out among the models that describe blood's rheological properties. It postulates that blood possesses a yield stress, acting as a solid up to a specific stress threshold before transitioning to a viscous liquid state upon surpassing this limit. Such a model provides a framework for understanding blood's behavior under physiological and pathological conditions. High blood viscosity is a concern as it can hinder efficient circulation and oxygen distribution to bodily tissues, which, in turn, exacerbates insulin resistance and the progression of DM (2,3). The interplay between blood rheology and DM underscores the potential of targeting blood viscosity as a therapeutic strategy.

Plant latex, a milky secretion produced by various plants, has been historically harnessed in traditional medicine for its myriad therapeutic properties, including wound healing and anti-inflammatory benefits. Among its active constituents, specific proteins within plant latex have been identified as modulators of the hemostatic and fibrinolytic systems – critical components of the body's mechanisms for blood coagulation and clot dissolution (Rajesh *et al.*, 2007; Venkatesha; Rajaiah; Vishwanath, 2016). Despite their known interactions with these systems, the influence of plant latex proteins on blood rheology, particularly in the context of diabetes mellitus (DM), remains underexplored. *Calotropis procera*, a xerophytic shrub belonging to the Apocynaceae family and thriving in the arid climates of Africa and Asia, is notable for its latex, which is rich in various bioactive

molecules. These include tannins, flavonoids, triterpenoids, steroids, and proteins, all credited with a broad spectrum of pharmacological activities such as antioxidant, anti-inflammatory, analgesic, antimicrobial, antimalarial, antidiabetic, wound-healing, hepatoprotective, neuroprotective, anti-ulcer, insecticidal, and anticancer effects (Dogara, 2023; Rajesh *et al.*, 2007). However, it is important to note that while offering potential health benefits, excessive consumption of Calotropis procera latex can lead to toxicity and adverse reactions (Rajesh *et al.*, 2007).

In light of these properties, our study investigates the potential benefits of *Calotropis procera* latex proteins (CpLP) on blood rheology in a diabetic mouse model subjected to acute inflammation. We measure the changes in rheological properties of blood following treatment with CpLP and assess any alterations in hematological parameters. We also evaluate the hematological parameters of the blood of treated mice. Our goal is to shed light on how CpLP influences blood viscosity, elucidating its possible implications for managing DM and enhancing vascular health.

4.2 Materials and Methods

4.2.1 Latex collection and processing

The latex of *C. procera* plants was collected by breaking the most apical internode of each shoot, the exuded latex was collected in tubes containing distilled water (1:1, v/v), which were centrifuged at 10,000 x g (4 ºC for 10 minutes). The supernatant, mostly rubber-free, was dialyzed against distilled water for 60 hours at 4 ºC, using dialysis membranes with 8 kDa of cutoff size. The sample was centrifuged again under the same conditions, and the supernatant, devoid of rubber and rich in proteins, was lyophilized to obtain soluble proteins, called CpLP (Alencar *et al.*, 2006).

4.2.2 Animal experimentation

Swiss mice were obtained from the Federal University of Ceara Central Animal House. All animal experimentation was executed by the Brazilian National Council of Control of Animal Experimentation (CONCEA) and previously approved by the Animal Care and Use Committee (CEUA) of the Federal University of Ceara under protocol 2742280720. To investigate the effects of CpLP on the immune system of diabetic animals, hyperglycemia was induced using a single high dose of Streptozotocin (180 mg/ kg, STZ), adapting from a published protocol (Furman, 2015). After 10 days, all animals with blood glucose greater than 220 mg/ dL were considered diabetic. Diabetic animals were treated with CpLP 30 mg/ kg, 24 hours before being infected with 10^4 Colony Forming Units per mL (CFU/ mL) of *Salmonella enterica* Typhimurium C5. Control animals received phosphate buffered saline (PBS). The blood of all animals was collected in EDTA and taken for hematological and rheological analysis.

4.2.3 Hematological analysis

The blood was drawn through the retroorbital plexus of anesthetized animals. Blood was collected in EDTA tubes. Blood vials were examined by a semi-automatic hematological analyzer, looking for red and white blood cells, hematocrit, platelets, and protein contents.

4.2.4 Rheological analysis

The blood rheology was analyzed using a stress-controlled rheometer (AR-550, TA Instruments) with a temperature controller and 40 mm 1°0'47" steel cone. The geometry gap was 27 µm. A solvent trap was used to avoid drying of the blood. The equipment was configured to get 10 points evenly spaced between the shear rate 0.1 and 10 Pascal. All rheology experiments were conducted at 37 ºC. To determine the rheological parameters of the whole blood, the rheogram curves were fitted to a generalized Bingham fluid model, also known as the Herschel-Bulkley equation. This model describes the rheologic behavior of complex fluids as follows:

$$\tau = \tau_0 + k\dot{\gamma}^n; \quad (1)$$

where τ is the shear stress and $\dot{\gamma}$ the shear rate, τ_0 the yield stress, i.e., the minimum stress required to initiate flow in the fluid, it reflects the strength of the internal bonds or interactions that hold the fluid together. A higher τ_0 leads to a more solid-like material. The consistency index of the fluid, k, reflects the overall flow resistance. Thus, a higher k means a more viscous or thick fluid. The flow behavior index n reflects the degree of nonlinearity of the stress and shear rate relationship. Fluids holding $n < 1$ present shear-thinning (or pseudoplastic) effects, where the viscosity decreases with increasing shear rate, while fluids with $n > 1$ present shear-thickening (or dilatant) behavior, and the viscosity increases with increasing shear rate. The traditional Bingham fluid model corresponds to the case where $n = 1$. Moreover, if $\tau_0 = 0$, Equation 1 recovers the classical Newtonian fluid equation the stress is proportional to the shear rate and the viscosity is constant regardless of the applied stress.

4.3 Results

4.3.1 Hematological results

The hematological analysis of blood samples from different groups of diabetic mice treated with PBS, CpLP, or CpLP + *Salmonella* 24 h was performed to evaluate the effects of these treatments on blood composition and function. The results indicate that the treatment with CpLP + *Salmonella* 24h increased the WBC count, hematocrit, platelets, and blood proteins compared to the PBS and CpLP groups. These changes suggest combining CpLP and Salmonella 24h induced inflammatory response and increased the clotting potential. The measured parameters for each group are summarized in Table 2 (excerpt from Table 1).

Table 2 - Hematological parameters of blood from diabetic mice infected with *Salmonella* and treated with *C. procera* latex proteins.

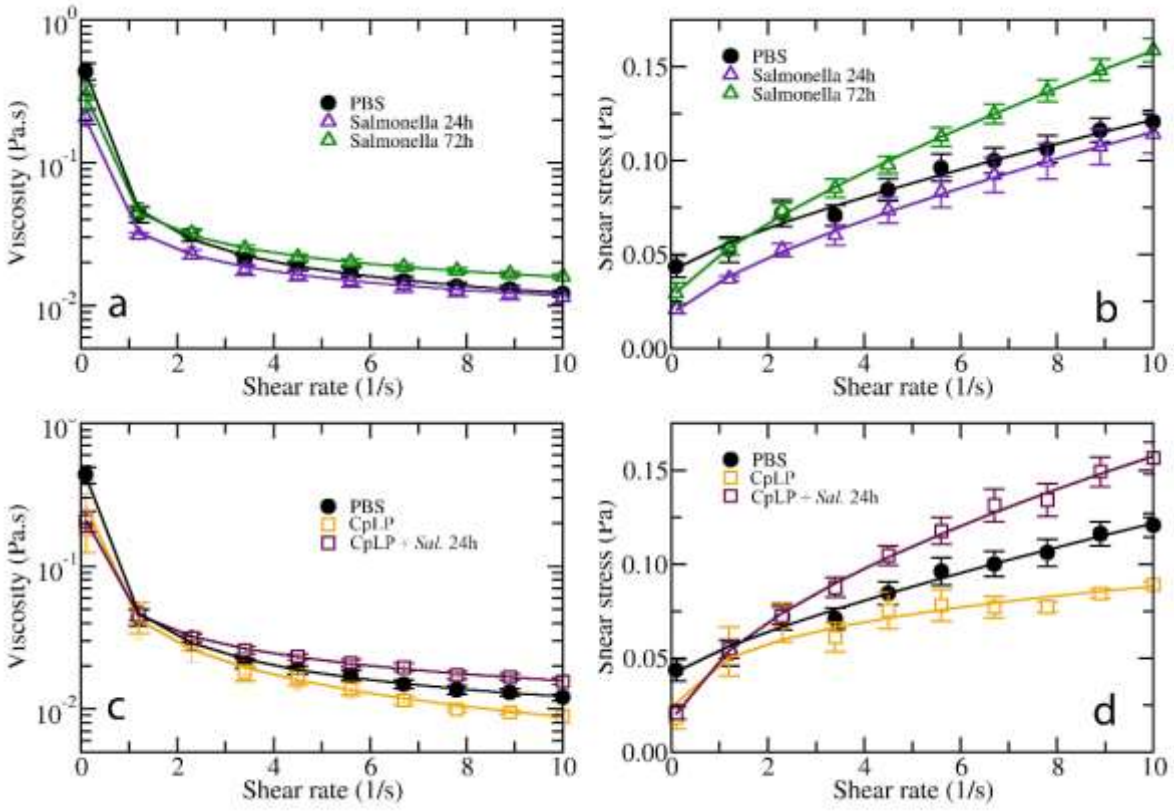
Blood Parameters	PBS	CpLP	CpLP + Sal. 24 h
RBC	9.78 ± 0.27	9.80 ± 0.26	9.89 ± 0.16
WBC	2.73 ± 0.18	3.33 ± 0.10	4.47 ± 0.14*
HC	46.67 ± 0.88	45.25 ± 1.55	48.20 ± 0.58
PLA	630.67 ± 30.14	623.00 ± 32.11	970.25 ± 37.08*
PRO	4.53 ± 0.29	4.75 ± 0.29	5.12 ± 0.12

Source: self-authorship
RBC: red blood cells (x10^6 cells/ µL). WBC: white blood cells (x10^3 cells/ µL). HC: hematocrit (%). PLA: platelets (x10^3 cells/ µL). PRO: proteins (g/ dL).
Values are expressed as mean ± standard error of the mean.
* represents statistical difference compared to PBS group. Two-Way ANOVA, $p<0.05$.

4.3.2 Rheological results

The investigation into the blood rheological behavior among various groups of hyperglycemic mice utilized the generalized Bingham fluid model as a foundational framework. This model considers that blood exhibits both a yield stress and a plastic viscosity, characteristics that are crucial for understanding how it behaves under stress. By applying such a model, the study aimed to accurately quantify these parameters, offering insights into the blood's rheological properties in diabetes mellitus (Figure 13).

Figure 13 - Mice rheological blood data from hyperglycemic mice infected with *Salmonella* and treated with *C. procera* latex proteins.



Source: self-authorship.
Mice rheological blood data for the different groups examined in a stress-controlled rheometer with a temperature of 37 ºC. Panels (a) and (c) show the rheogram curves, shear stress (σ) measures in Pa, and shear rate (γ) in 1/s. Panels (b) and (d) show how the viscosity for each group changes with the shear rate—the viscosity (η) given in Pa.s. The mean values are computed for different samples (symbols) with their respective standard errors (bars). Solid lines represent the fit of measured data with the Bingham fluid behavior to calculate blood rheological parameters (shown in Table 3).

The parameters for the non-diabetic case were obtained from a different work done by our research group (Silva *et al.*, 2019). We find a pronounced effect of hyperglycemia on blood rheology, notably increasing the yield stress and the flow behavior index. This alteration suggests that diabetes might render the blood more rigid and prone to shear-thinning, deviating from the ideal fluidity needed for healthy vascular function. In contrast, infection with *Salmonella* appears to mitigate these diabetes-induced changes, decreasing both the yield stress and the flow behavior index, thereby enhancing the blood's fluidity, and reducing its shear-thinning behavior compared to the diabetic PBS-treated group (Table 3).

Table 3 - Generalized Bingham fluid rheology fit data of blood from hyperglycemic mice infected with *Salmonella* and treated with *C. procera* latex proteins.

Groups	**Yield stress (τ_0)**	**Consistency index (k)**	**Flow behavior index (n)**
Non-diabetic	0.0	0.07	0.53
PBS	0.04	0.01	0.77
***Salmonella* 24h**	0.02	0.02	0.71
***Salmonella* 72h**	0.03	0.03	0.73
CpLP	0.0	0.05	0.27
CpLP + *Sal.* 24h	0.01	0.04	0.57

Source: non-diabetic group was taken from (Silva *et al.*, 2019).
The remaining data is self-authored.

Interestingly, treatment with CpLP also reduced yield stress and flow behavior index, although to a lesser degree than observed with Salmonella infection. This result indicates that CpLP moderates the rheological changes induced by diabetes, promoting improved blood fluidity. However, when CpLP treatment was combined with *Salmonella* infection, there was an increase in both yield stress and flow behavior index relative to the CpLP-alone group, suggesting an interactive effect that partially counteracts the fluidity-enhancing properties of CpLP. Despite these interactions, the combined treatment did not revert rheological parameters to the levels observed in the diabetic PBS group, indicating a complex interplay between diabetes, infection, and CpLP treatment in modulating blood rheology. These results underscore the significance of understanding blood rheology in the context of

diabetes and the potential of CpLP as a therapeutic agent to improve blood flow characteristics, with implications for managing diabetes and its vascular complications.

4.4 Discussion

The investigation into the dynamics of blood rheology within the context of diabetes mellitus (DM) underscores the intricate interplay between metabolic disorders and vascular health. By focusing on the effects of *Calotropis procer*a latex proteins (CpLP) on blood rheology and hematology in diabetic mice, our study illuminates the potential of natural compounds in modulating blood characteristics to enhance vascular functionality.

DM is known to alter the rheological properties of blood, including viscosity, elasticity, and cell aggregation, contributing to impaired blood flow and increased cardiovascular risk (Irace *et al.*, 2014; Sun *et al.*, 2022; Tamariz *et al.*, 2008). The oxidative stress, inflammation, and endothelial dysfunction induced by DM exacerbate these rheological changes, promoting vascular damage and diseases such as atherosclerosis, thrombosis, and ischemia (Kuller *et al.*, 2000). Our findings suggest that CpLP can reduce the yield stress and flow behavior index in hyperglycemic mice, indicating an improvement in blood fluidity and shear-thinning behavior. Such modifications have significant implications for preventing and treating diabetic complications, given the crucial role of shear thinning in enhancing blood flow efficiency, oxygen delivery, and nutrient transport (Valeanu; Ginghina; Bubenek-Turconi, 2021).

The study further explores the physiological basis of blood's shear-thinning properties, primarily attributed to the deformability and orientation of red blood cells under varying shear rates. This rheological feature is essential for minimizing energy dissipation within blood vessels, especially in microcirculation where high shear rates prevail (Nader *et al.*, 2019; Sriram; Intaglietta; Tartakovsky, 2014). Additionally, shear thinning influences nitric oxide production by endothelial cells, a critical factor in vascular tone regulation (Kelly *et al.*, 2020), and impacts hemostasis and thrombosis through its effects on platelet activity and fibrin dynamics (Singh; Singh, 2024).

However, DM-induced changes in red blood cell deformability and aggregation can disrupt these beneficial shear-thinning properties, increasing blood

viscosity and cardiovascular complications (Sun *et al.*, 2022). Our findings, demonstrating CpLP ability to mitigate these changes, align with existing research on plant latex proteins' role in modulating blood rheology and hemodynamics. For example, *Ficus carica* latex proteins have improved blood flow in mice with arterial hypertension (Alamgeer *et al.*, 2017). *Carica papaya* latex proteins have been shown to reduce platelet aggregation in mice with hypercholesterolemia (Munir *et al.*, 2022). *Pergularia daemia* latex proteins have been shown to enhance blood circulation and glycemia in mice with diabetes (Kumar; Ramesh; Vinoth Kumar, 2014). These studies suggest that plant latex proteins can act as natural anticoagulants and vasodilators and may have potential applications in treating cardiovascular diseases.

These results suggest that CpLP and similar compounds could serve as valuable therapeutic agents in managing vascular diseases associated with DM. Noteworthy is the consideration that varying experimental conditions, such as temperature and rheometer geometry, significantly influence rheological assessments. Our adoption of experimental setups that more closely mimic physiological blood flow conditions ensures the pertinence and reliability of our findings, distinguishing them from other studies that might not accurately capture the in vivo rheological behavior of blood.

Additionally, CpLP effects extend to hematological parameters, eliciting changes indicative of an inflammatory response and augmented clotting potential. These hematological and rheological enhancements collectively underscore CpLP prospective benefits for vascular health in diabetic scenarios. Therefore, the rheological and hematological data obtained in this study are consistent and complementary, as they both show the effects of CpLP on blood rheology and hematology in diabetic mice infected with *Salmonella*.

4.5 Conclusion

We investigated the effects of *Calotropis procera* latex protein on blood rheology and hematology in hyperglycemic mice. This protein significantly improved blood viscosity and hematological parameters compared to the control group, suggesting a beneficial impact on blood flow, often compromised by hyperglycemia and oxidative stress in diabetes. Additionally, our findings suggest that the latex protein may modulate the immunological response of blood cells. This study enhances our understanding of blood rheology in diabetes mellitus (DM). It identifies CpLP as a promising natural therapeutic candidate for improving blood properties and vascular health in diabetic individuals. To our knowledge, this is one of the first studies to illustrate the effect of *C. procera* latex protein on blood rheology and hematology in hyperglycemic mice, adding to the evidence that supports *C. procera*'s potential as an anti-diabetic agent. Moreover, our work reveals a new dimension to the pharmacological properties of plant latex proteins, traditionally recognized for their anti-inflammatory, antimicrobial, and coagulant activities. Future research should delve into the mechanisms of CpLP's action and its clinical relevance, which will be crucial for harnessing its full potential in treating cardiovascular diseases related to diabetes. Our findings encourage further investigation into the molecular mechanisms by which latex protein influences blood rheology and hematology, its impact on other biochemical and physiological diabetes-related parameters, and the assessment of its safety and efficacy in future trials relative to conventional anti-diabetic medications.

5 GENERAL CONCLUSION

The comprehensive investigation presented in this dissertation has shed light on the multifaceted role of *Calotropis procera* latex protein in the context of diabetes mellitus. Our research has traversed the realms of immunomodulation, cytokine response, and blood rheology to uncover the therapeutic potential of CpLP in hyperglycemic conditions.

From the immunological perspective, CpLP treatment demonstrated a remarkable ability to balance pro-inflammatory and anti-inflammatory responses, thereby reducing inflammatory damage while preserving the host's defense against bacterial infection. Histopathological analyses further revealed the nuanced effects of CpLP on various organs, indicating an amplified immune response and organ-specific reactions.

In terms of blood rheology, CpLP emerged as a promising agent for improving blood viscosity and hematological parameters, which are often adversely affected by hyperglycemia and oxidative stress in DM. The modulation of the immunological response of blood cells by CpLP points to its potential in enhancing vascular health and blood flow, crucial aspects that are compromised in diabetic individuals.

The convergence of these findings underscores CpLP's promise as a natural therapeutic candidate with a broad spectrum of pharmacological properties. This dissertation not only contributes to the existing body of knowledge on the anti-diabetic effects of plant latex proteins but also opens new avenues for exploring their role in cardiovascular health.

As we look to the future, it is imperative to delve deeper into the molecular mechanisms underlying CpLP's action, its interaction with blood components, and its overall impact on the diabetic milieu. The safety, efficacy, and clinical relevance of CpLP must be rigorously assessed in comparison to conventional anti-diabetic medications, paving the way for novel interventions in the management of DM and its associated cardiovascular complications.

In conclusion, this dissertation affirms the potential of *Calotropis procera* latex protein as an innovative and natural therapeutic avenue for diabetes management, with implications that extend beyond glycemic control to encompass immunological balance and vascular integrity.

REFERENCES

AKIN, Seydahmet; BÖLÜK, Cem. Prevalence of comorbidities in patients with type–2 diabetes mellitus. **Primary Care Diabetes**, [*s. l.*], v. 14, n. 5, p. 431–434, 2020.

ALAM, Safaet *et al.* **Antidiabetic Phytochemicals From Medicinal Plants: Prospective Candidates for New Drug Discovery and Development**. [*S. l.*]: Frontiers Media S.A., 2022.

ALAM, Saruar *et al.* Diabetes Mellitus: Insights from Epidemiology, Biochemistry, Risk Factors, Diagnosis, Complications and Comprehensive Management. **Diabetology**, [*s. l.*], v. 2, n. 2, p. 36–50, 2021.

ALAMGEER *et al.* Evaluation of antihypertensive potential of Ficus carica fruit. **Pharmaceutical Biology**, [*s. l.*], v. 55, n. 1, p. 1047–1053, 2017.

ALENCAR, Nylane Maria Nunes *et al.* Pro- and anti-inflammatory activities of the latex from Calotropis procera (Ait.) R.Br. are triggered by compounds fractionated by dialysis. **Inflammation Research**, [*s. l.*], v. 55, n. 12, p. 559–564, 2006.

ALVES, Cresio; CASQUEIRO, Juliana; CASQUEIRO, Janine. Infections in patients with diabetes mellitus: A review of pathogenesis. **Indian Journal of Endocrinology and Metabolism**, [*s. l.*], v. 16, n. 7, p. 27, 2012.

AMARAL, Jackson L. *et al.* Peptide fraction from latex of Calotropis procera exhibits antifungal and insecticidal activities. **Arthropod-Plant Interactions**, [*s. l.*], v. 15, n. 3, p. 431–438, 2021.

AMERICAN DIABETES ASSOCIATION. Pharmacologic Approaches to Glycemic Treatment: Standards of Medical Care in Diabetes - 2020. **Diabetes Care**, [*s. l.*], v. 43, n. Supplement_1, p. S98–S110, 2020.

AMINI, Mohammad Humayoon *et al.* Important insights from the antimicrobial activity of Calotropis procera. **Arabian Journal of Chemistry**, [*s. l.*], v. 14, n. 7, p. 103181, 2021.

BEZERRA, Camila F. *et al.* Latex proteins from Calotropis procera: Toxicity and immunological tolerance revisited. **Chemico-Biological Interactions**, [*s. l.*], v. 274, p. 138–149, 2017.

BLAHOVA, Jana *et al.* **Pharmaceutical drugs and natural therapeutic products for the treatment of type 2 diabetes mellitus**. [*S. l.*]: MDPI, 2021.

CIASCA, G. *et al.* Mapping viscoelastic properties of healthy and pathological red blood cells at the nanoscale level. **Nanoscale**, [*s. l.*], v. 7, n. 40, p. 17030–17037, 2015.

DE OLIVEIRA, Keciany Alves *et al.* Phytomodulatory proteins promote inhibition of hepatic glucose production and favor glycemic control via the AMPK pathway.

Biomedicine & Pharmacotherapy, [*s. l.*], v. 109, n. November 2018, p. 2342–2347, 2019.

DINARELLO, Charles A. Proinflammatory Cytokines*. **American College of Chest Physicians**, [*s. l.*], 2000.

DOGARA, Abdulrahman Mahmoud. A systematic review on the biological evaluation of Calotropis procera (Aiton) Dryand. **Future Journal of Pharmaceutical Sciences**, [*s. l.*], v. 9, n. 1, 2023.

FREITAS, Cleverson D T *et al.* First insights into the diversity and functional properties of chitinases of the latex of Calotropis procera. **Plant Physiology and Biochemistry**, [*s. l.*], v. 108, p. 361–371, 2016.

FREITAS, Cleverson Diniz Teixeira *et al.* Osmotin from Calotropis procera latex: New insights into structure and antifungal properties. **Biochimica et Biophysica Acta - Biomembranes**, [*s. l.*], v. 1808, n. 10, p. 2501–2507, 2011.

FREITAS, Cleverson Diniz Teixeira. **Proteínas do Látex de Calotropis procera (Ait.) R. Br. e Seus Efeitos Sobre Pragas Agrícolas**. 2006. 118 f. [*s. l.*], 2006.

FURMAN, Brian L. Streptozotocin-Induced Diabetic Models in Mice and Rats. **Current Protocols in Pharmacology**, [*s. l.*], v. 70, n. 1, p. 5.47.1-5.47.20, 2015.

FURMAN, Brian L. Streptozotocin-Induced Diabetic Models in Mice and Rats. **Current Protocols**, [*s. l.*], v. 1, n. 4, 2021.

GEERLINGS, Suzanne E; HOEPELMAN, Andy I.M. Immune dysfunction in patients with diabetes mellitus (DM). **FEMS Immunology & Medical Microbiology**, [*s. l.*], v. 26, n. 3–4, p. 259–265, 1999.

GUERRA, M. P.; NODARI, R.O. **Biodiversidade: aspectos biológicos, geográficos, legais e éticos**. 5. ed. Porto Alegre: [*s. n.*], 2004.

GUZIK, T. J.; KORBUT, R.; ADAMEK-GUZIK, T. Nitric oxide and superoxide in inflammation and immune regulation. **Journal of Physiology and Pharmacology**, [*s. l.*], v. 54, n. 4, p. 469–487, 2003.

IRACE, Concetta *et al.* Blood viscosity in subjects with normoglycemia and prediabetes. **Diabetes Care**, [*s. l.*], v. 37, n. 2, p. 488–492, 2014.

JONG, H. K. *et al.* Host-pathogen interaction in invasive Salmonellosis. **PLos Pathog.**, [*s. l.*], v. 8, n. 10, 2012.

KELLY, Nathaniel S. *et al.* Influence of shear-thinning blood rheology on the laminar-turbulent transition over a backward facing step. **Fluids**, [*s. l.*], v. 5, n. 2, 2020.

KING, Aileen JF. The use of animal models in diabetes research. **British Journal of Pharmacology**, v. 166, n. 3, p. 877–894, 2012.

KULLER, Lewis H *et al.* **Diabetes Mellitus Subclinical Cardiovascular Disease and Risk of Incident Cardiovascular Disease and All-Cause Mortality**. [*S. l.: s. n.*], 2000.

KUMAR, V. L. *et al.* Protective effect of proteins derived from Calotropis procera latex against acute inflammation in rat. **Autonomic and Autacoid Pharmacology**, [*s. l.*], v. 35, n. 1–2, p. 1–8, 2015.

KUMAR, Vijay L. *et al.* Protective Effect of Proteins Derived from the Latex of Calotropis procera against Inflammatory Hyperalgesia in Monoarthritic Rats. **Phytotherapy Research**, [*s. l.*], v. 35, n. 1–2, p. n/a-n/a, 2011.

KUMAR, P Vinoth; RAMESH, N; VINOTH KUMAR, P. **Anti-hyperglycaemic activity of Pergularia daemia (Forssk.) ChiovThe Journal of Phytopharmacology**. [*S. l.: s. n.*], 2014.

KUMAR, V. et al. Protective Effect of Proteins Derived from Seed Explant Cultures of Calotropis procera in a Preclinical Model of Arthritis. **Planta medica letters**, v. 2, n. 01, p. e52–e56, 2015.

LENZEN, S. **The mechanisms of alloxan- and streptozotocin-induced diabetes**. [*S. l.: s. n.*], 2008.

LEWIS, Steven M.; WILLIAMS, Adam; EISENBARTH, Stephanie C. Structure and function of the immune system in the spleen. **Science Immunology**, [*s. l.*], v. 4, n. 33, 2019.

LI, Feng *et al.* Expert Opinion on Drug Discovery Are we seeing a resurgence in the use of natural products for new drug discovery ?. **Expert Opinion on Drug Discovery**, [*s. l.*], v. 14, n. 5, p. 417–420, 2019.

LIMA-FILHO, J. V.M. *et al.* Effect of the Escherichia coli EMO strain on experimental infection by Salmonella enterica serovar Typhimurium in gnotobiotic mice. **Brazilian Journal of Medical and Biological Research**, [*s. l.*], v. 37, n. 7, p. 1005–1013, 2004.

LIMA-FILHO, José V. *et al.* Proteins from latex of Calotropis procera prevent septic shock due to lethal infection by Salmonella enterica serovar Typhimurium. **Journal of Ethnopharmacology**, [*s. l.*], v. 129, n. 3, p. 327–334, 2010.

LIU, Guiming; DANESHGARI, Firouz. **Diabetic Bladder Dysfunction HHS Public AccessChin Med J (Engl)**. [*S. l.: s. n.*], 2014.

MUIR, Jennifer A.; ESPINAS, Marites; DELABIE, Jan. Localized lymphoid hyperplasia of the spleen: a rare benign condition grossly mimicking malignancy. **Journal of Hematopathology**, [*s. l.*], v. 9, n. 2, p. 85–89, 2016.

MUNIR, S. et al. Delving into the Therapeutic Potential of Carica papaya Leaf against Thrombocytopenia. **Molecules**, v. 27, n. 9, p. 2760, 2022.

MUSHTAQ, Mazhar; ABDUL MATEEN, Muhammad; KIM, Uh Hyun. Hyperglycemia associated blood viscosity can be a nexus stimuli. **Clinical Hemorheology and Microcirculation**, [*s. l.*], v. 71, n. 1, p. 103–112, 2019.

NADER, E. et al. Blood Rheology: Key Parameters, Impact on Blood Flow, Role in Sickle Cell Disease and Effects of Exercise. **Frontiers in Physiology**, v. 10, n. 10, 2019.

OBESE, Ernest *et al.* Antinociceptive Effect of The Hydroethanolic Leaf Extract of Calotropis procera (Ait) R. Br. (Apocynaceae): possible involvement of glutamatergic, cytokines, opioidergic and adenosinergic pathways. **Journal of Ethnopharmacology**, [*s. l.*], v. 278, n. May, p. 114261, 2021.

OLIVEIRA, Raquel S.B. *et al.* Inflammation induced by phytomodulatory proteins from the latex of Calotropis procera (Asclepiadaceae) protects against Salmonella infection in a murine model of typhoid fever. **Inflammation Research**, [*s. l.*], v. 61, n. 7, p. 689–698, 2012.

ORGANIZATION, World Health. **No Title**. [*S. l.*], 2021. Disponível em: https://www.who.int/health-topics/diabetes. Acesso em: 15 fev. 2021.

PATEL, Roshni *et al.* Amelioration of complete Freund's adjuvant-induced arthritis by Calotropis procera latex in rats. **Future Journal of Pharmaceutical Sciences**, [*s. l.*], v. 7, n. 1, 2021.

PELEG, A. Y. et al. Common infections in diabetes: pathogenesis, management and relationship to glycaemic control. **Diabetes/Metabolism Research and Reviews**, v. 23, n. 1, p. 3–13, 2006.

POLL, Tom van der *et al.* The immunopathology of sepsis and potential therapeutic. **Nature Reviews Immunology**, [*s. l.*], v. 17, p. 407–420, 2017.

PORTILLO, F. G. Salmonella intracellular proliferation: where, when and how?. **Microbes and infection**, [*s. l.*], v. 3, p. 1305–1311, 2001.

RAJESH, Rajaiah *et al.* Comparative Study on Plant Latex Proteases and their Involvement in Hemostasis: A Special Emphasis on Clot Inducing and Dissolving Properties. **Planta Medica**, [*s. l.*], v. 73, n. 10, p. 1061–1067, 2007.

RAMOS, Márcio V. *et al.* Anti-inflammatory latex proteins of the medicinal plant Calotropis procera: a promising alternative for oral mucositis treatment. **Inflammation Research**, [*s. l.*], v. 69, n. 9, p. 951–966, 2020.

RAMOS, Márcio Viana *et al.* Laticifers, Latex, and Their Role in Plant Defense. **Trends in Plant Science**, [*s. l.*], v. 24, n. 6, p. 553–567, 2019.

ROSCHE, Kristin L. *et al.* Infection with Salmonella enterica serovar typhimurium leads to increased proportions of F4/80+ red pulp macrophages and decreased proportions of B and T lymphocytes in the spleen. **PLoS ONE**, [*s. l.*], v. 10, n. 6, 2015.

SANTOS, R. L. *et al.* Pathogenesis of Salmonella-induced enteritis. **Brazilian Journal of Medical and Biological Research**, [*s. l.*], v. 36, n. 1, p. 3–12, 2003.

SILVA, Ayrles F B *et al.* Erythrocytes morphology and hemorheology in severe bacterial infection. **Memórias do Instituto Oswaldo Cruz**, [*s. l.*], v. 114, n. 11, p. 1–8, 2019.

SINGH, Damini; SINGH, Sarita. Computational analysis of patient-specific pulsatile blood flow: The influence of non-Newtonian models on wall shear stress assessment. **Physics of Fluids**, [*s. l.*], v. 36, n. 1, 2024.

SOUSA, Brandon Ferraz *et al.* Latex proteins downregulate inflammation and restores blood-coagulation homeostasis in acute Salmonella infection. **Memórias do Instituto Oswaldo Cruz**, [*s. l.*], v. 115, n. 11, p. 1–12, 2020.

SOUTO, Patrícia Carneiro *et al.* Biometria de frutos e número de sementes de Calotropis procera (Ait.) R. Br. no semi-árido da Paraiba. **Revista Verde de Agroecologia e Desenvolvimento Sustentável**, [*s. l.*], v. 3, n. 1, 2008.

SRIRAM, Krishna; INTAGLIETTA, Marcos; TARTAKOVSKY, Daniel M. Non-Newtonian Flow of Blood in Arterioles: Consequences for Wall Shear Stress Measurements. **Microcirculation**, [*s. l.*], v. 21, n. 7, p. 628–639, 2014.

SUN, Jiehui *et al.* Blood Viscosity in Subjects With Type 2 Diabetes Mellitus: Roles of Hyperglycemia and Elevated Plasma Fibrinogen. **Frontiers in Physiology**, [*s. l.*], v. 13, 2022.

SZKUDELSKI, T. The Mechanism of Alloxan and Streptozotocin Action in B Cells of the Rat Pancreas. **Physiology Research**, [*s. l.*], 2001.

TAMARIZ, Leonardo J. *et al.* Blood viscosity and hematocrit as risk factors for type 2 diabetes mellitus: The Atherosclerosis Risk in Communities (ARIC) Study. **American Journal of Epidemiology**, [*s. l.*], v. 168, n. 10, p. 1153–1160, 2008.

TAVARES, Lethicia Souza *et al.* Perspectives for the use of latex peptidases from Calotropis procera for control of inflammation derived from Salmonella infections. **International Journal of Biological Macromolecules**, [*s. l.*], v. 171, p. 37–43, 2021.

TO, Cells *et al.* Salmonella enterica Serovar Typhimurium Exploits Cycling Enteroids. [*s. l.*], v. 12, n. 1, p. 1–18, 2021.

TONIOLO, Antonio *et al.* The diabetes pandemic and associated infections: Suggestions for clinical microbiology. **Reviews in Medical Microbiology**, [*s. l.*], v. 30, n. 1, p. 1–17, 2019.

TRACEY, Kevin J. The inflammatory reflex. **Nature**, [*s. l.*], 2002.

TRAN, Ngan; PHAM, Bao; LE, Ly. **Bioactive compounds in anti-diabetic plants: From herbal medicine to modern drug discovery**. [*S. l.*]: MDPI AG, 2020.

TRIVEDI, Vatsal; LALU, Manoj M. **Handbook of Sepsis**. [*S. l.*]: Springer International Publishing, 2018-. ISSN 0003-2999.

VALEANU, Liana; GINGHINA, Carmen; BUBENEK-TURCONI, Serban. Blood Rheology Alterations in Patients with Cardiovascular Diseases. **Romanian journal of anaesthesia and intensive care**, [*s. l.*], v. 28, n. 2, p. 41–46, 2021.

VAN DER POLL, T.; OPAL, S. M. Host–pathogen interactions in sepsis. **The Lancet Infectious Diseases**, v. 8, n. 1, p. 32–43, 2008.

VENKATESHA, Shivaprasad H; RAJAIAH, Rajesh; VISHWANATH, Bannikuppe S. Hemostatic Interference of Plant Latex Proteases. **SM Journal of Clinical Pathology**, [*s. l.*], v. 1, n. 1002, 2016.

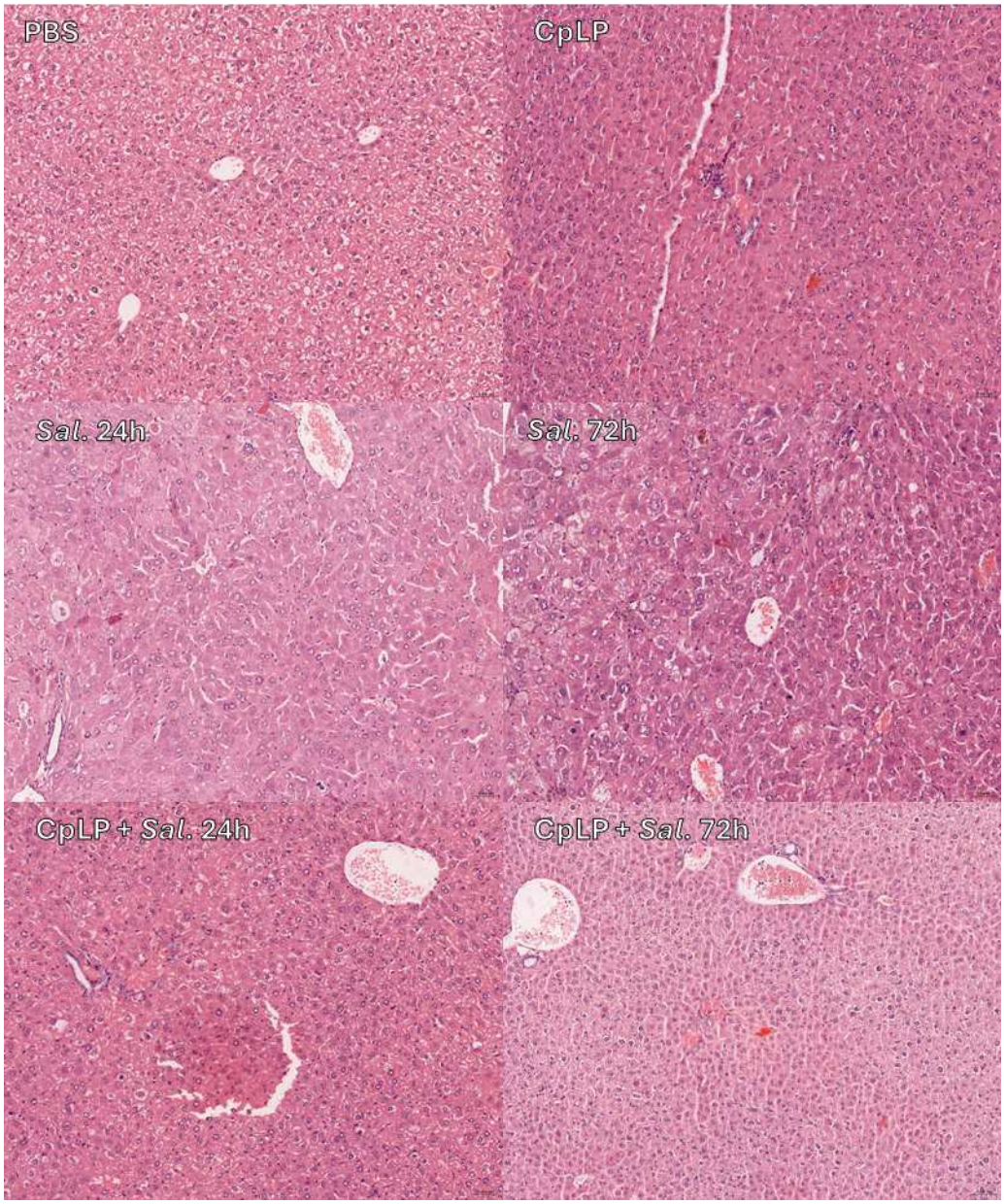
VERMA, S R. **Mathematical Modeling of Bingham Plastic Model of Blood Flow Through Stenotic VesselJournal of Engineering Research and Applications www.ijera.com**. [*S. l.: s. n.*], 2014.

YANG, Hua; WRIGHT, James R. **Human Cells Are Exceedingly Resistant to Streptozotocin in Vivo**. [*S. l.: s. n.*], 2002.

YANIV, Zohara; KOLTAI, Hinanit. Calotropis procera, Apple of Sodom. **Israel Journal of Plant Sciences**, [*s. l.*], 2018.

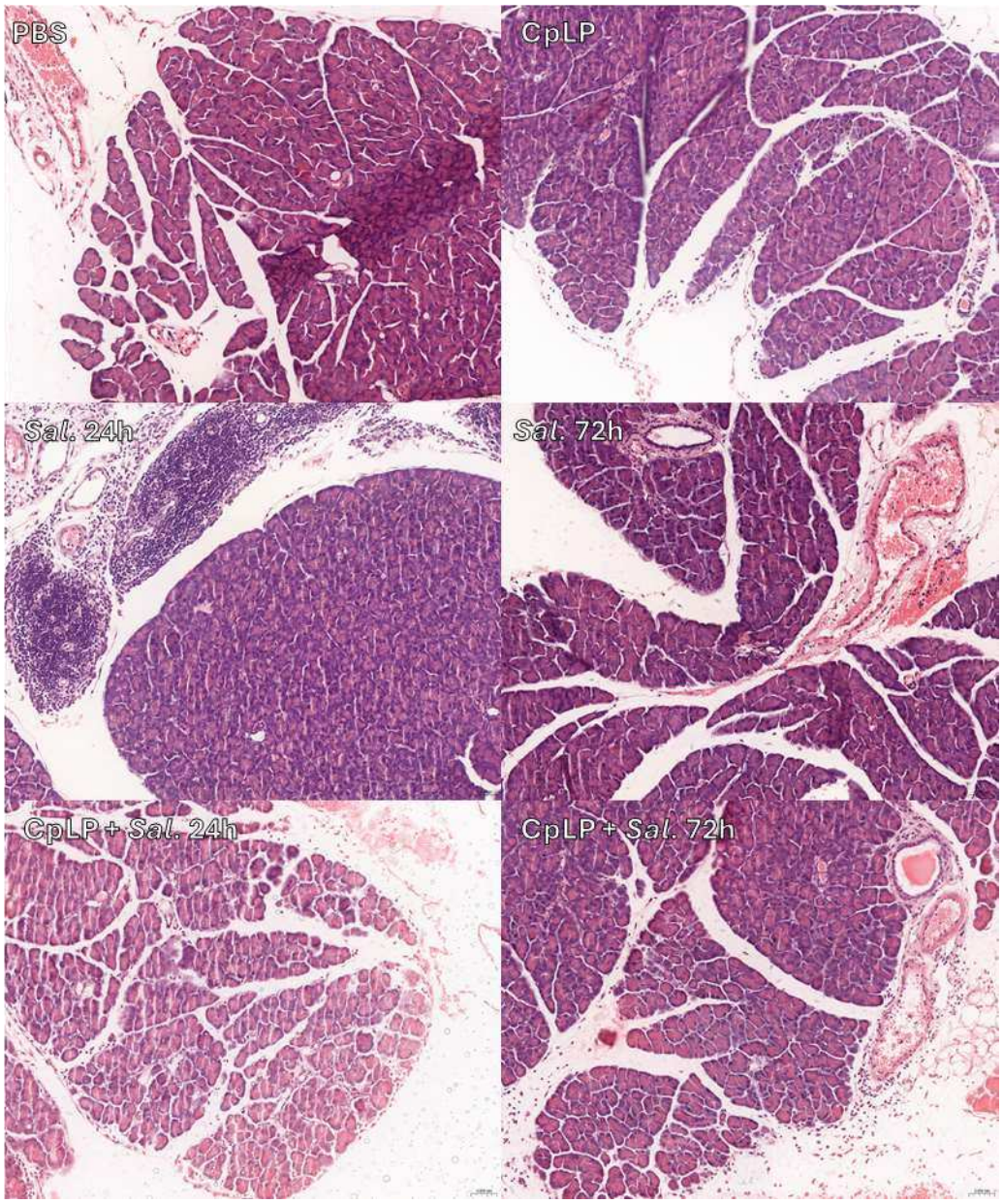
ZHANG, Xueping *et al.* Unraveling the regulation of hepatic gluconeogenesis. **Frontiers in Endocrinology**, [*s. l.*], v. 10, n. JAN, p. 1–17, 2019.

APPENDIX A – HISTOPATHOLOGICAL IMAGES OF LIVER



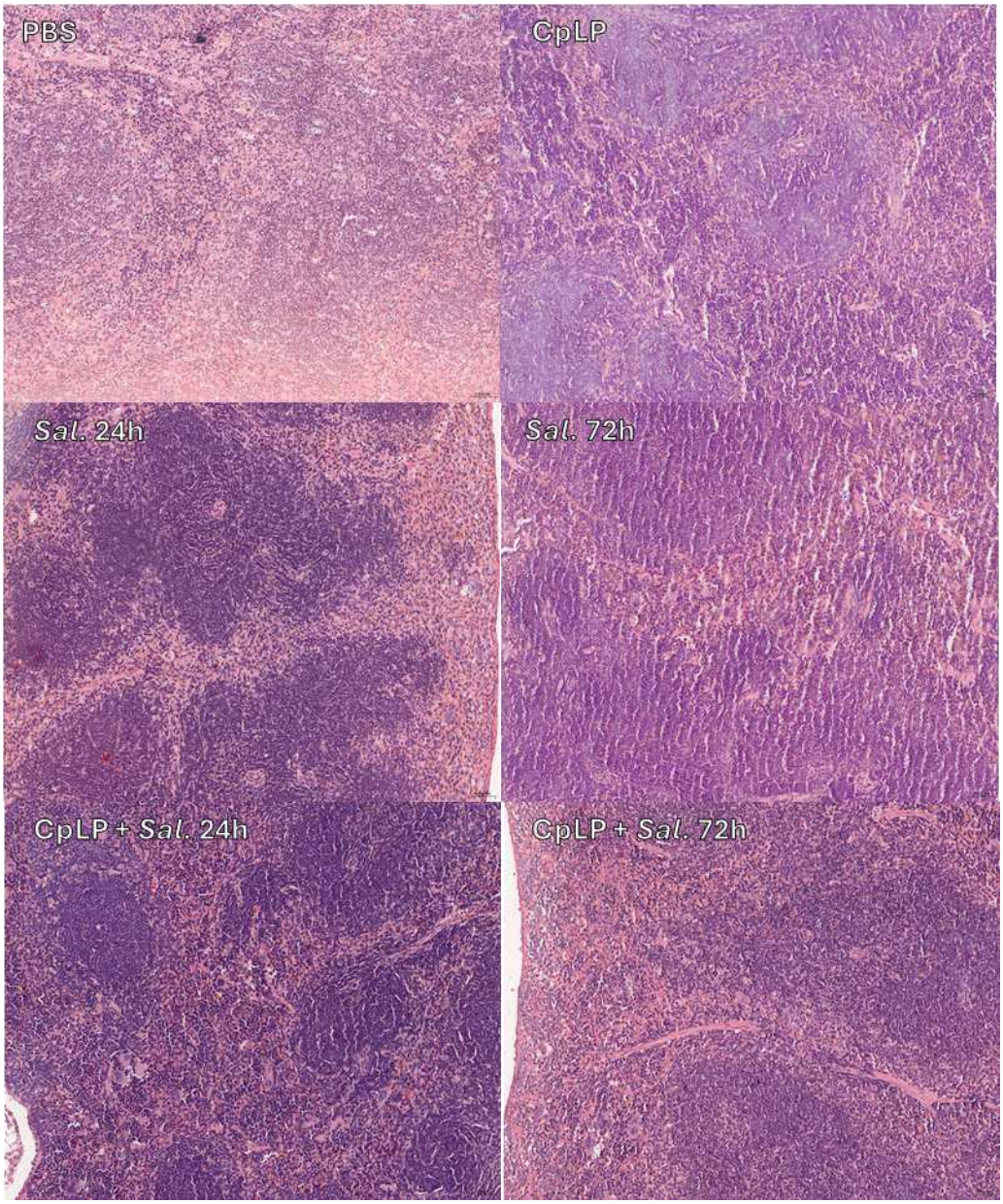
Source: self-authorship.

APPENDIX B – HISTOPATHOLOGICAL IMAGES OF PANCREAS



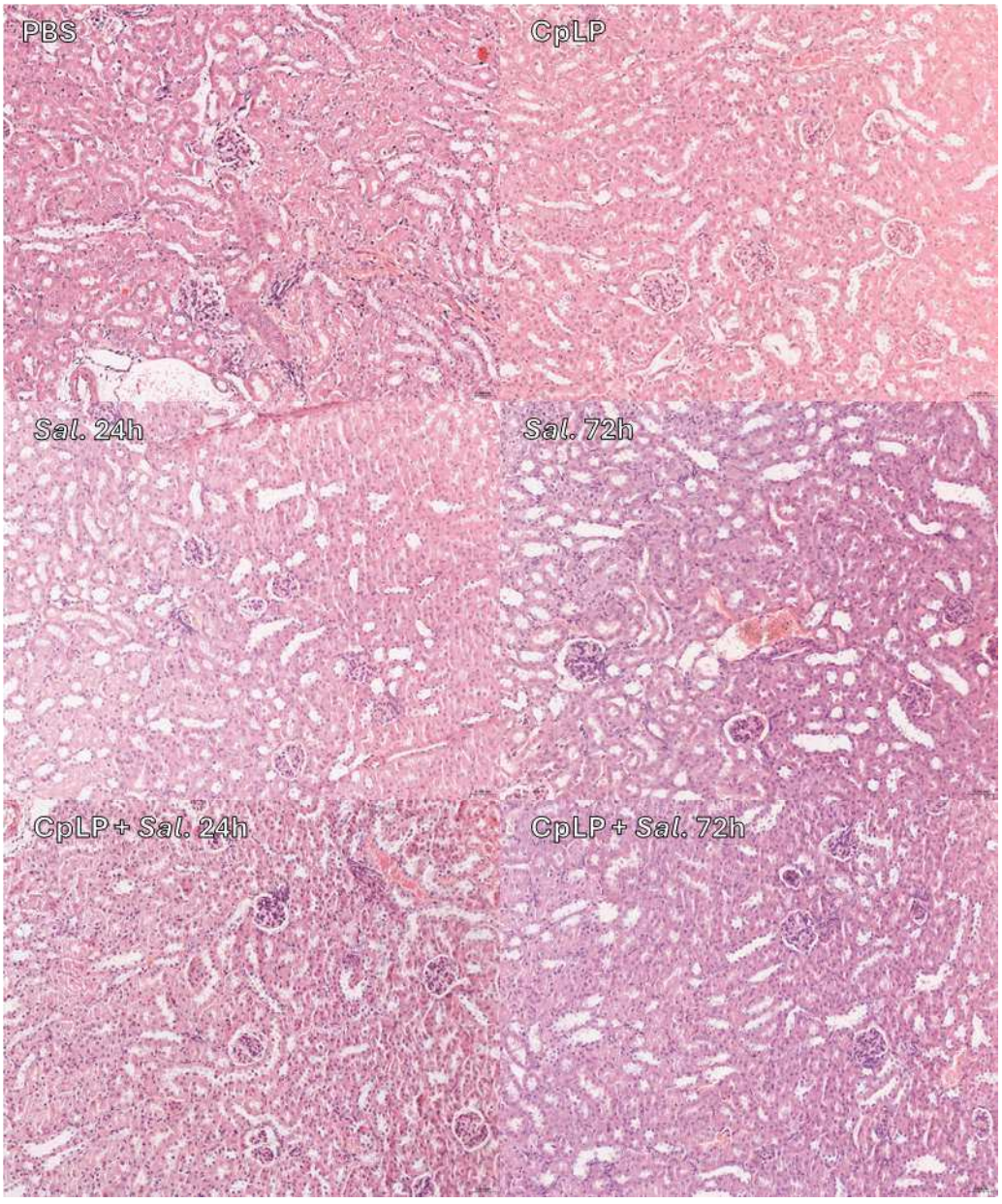
Source: self-authorship.

APPENDIX C – HISTOPATHOLOGICAL IMAGES OF SPLEEN



Source: self-authorship.

APPENDIX D – HISTOPATHOLOGICAL IMAGES OF KIDNEYS



Source: self-authorship